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Artemia Reference Center

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Effects of environmental factors on cyst hatching and on larval quality of the brine shrimp *Artemia sp.* (Anostraca: Branchiopoda)

Thesis submitted in fulfilment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences (Aquaculture)

“To my family for their patience, sacrifice and unshakable support”

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List of abbreviations and units

°C	Degree Celsius
%	Percentage
/	Per
±	Approximately
Δ	Isotopic discrimination
β-glucans	Beta-glucans
A	Analog
ADP	Adenosinedyphosphate
AFDW	Ash free dry weight
AMP	Adenosinemonophosphate
ANOVA	Analysis of variance
ARA	Arachidonic acid
ARC	*Artemia* Reference Center
At	Atom
ATP	Adenosinetryphosphate
BY	Bolshoye Yarovoye Lake, Siberia
C	Carbon
CFU	Colony forming unit
CL	Continuous light
cm	Centimeter
D	Digital
d.d.f.	Denominator degree of freedom
DHA	Docosahexaenoic acid
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DT	*Dunaliella tertiolecta*
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
EFA	Essential fatty acids
EPA	Eicosapentaenoic acid
EUROSCARF	European *Saccharomyces cerevisiae* Archive for Functional Analysis, Germany
FA	Fatty acid
FAME	Fatty acid methyl ester
FAO	Food and Agricultural Organization of the United Nations
FASW	Filtered autoclaved seawater
FIOSW	Filtered Instant Ocean seawater
g	Gram
GART	Gnotobiotic *Artemia* test system
GC	Gas chromatograph
h	Hour
H%	Hatching percentage
H_2O_2	Hydrogen peroxide
H/D	Hydration/dehydration
HGLMM	Hierarchical linear mixed model
HPLC	High-performance liquid chromatography
Hsps	Heat shock proteins
HUFAs	High unsaturated fatty acids
IAA	Isoascorbic acid
j	Joule
L	Liter
LEAPs	Late embryogenesis abundant proteins

LMM	Linear mixed model
LSD	Least significant differences
LVS....	Strain....isolated By Laurent Verschuere
m^2	Square meter
MA	Marine agar 2216
mg	Milligram
$MgCl_2$	Magnesium chloride
min	Minute
mj	Millijoule
ml	Milliliter
mm	Millimeter
Mnn....	Mutants with deleted genes involved in the extension of mannose in cell wall proteins
mRNA	Messenger ribonucleic acid
MSE	Mean square of groups
MUFA	Monounsaturated fatty acids
n	Number of replicates
n/a	Not applied
NaCl	Sodium chloride
NaOCl	Sodium hypochlorite
NaOH	Sodium hydroxide
$Na_2S_2O_35H_2O$	Sodium thiosulphate pentahydrate
NaH_2PO_4	Sodium dihydrogen phosphate
n.d.f.	Numerator degree of freedom
nm	Nanometer
no.	Number
NO	Nitric oxide
OD	Optical density
p	Statistical p-value obtained
PCR	Polymerase chain reaction
pH	Measure of the acidity of a solution
ppt	Parts per thousand
pr	Probability
PSEM	Pooled standard error of means
PUFA	Polyunsaturated fatty acids
REML	Residual maximum likelihood
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rotations per minute
s	Second
SDS-page	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFA	Saturated fatty acids
SFB	San Francisco Bay, USA
SPSS	Statistical package for the social science
TZ	Tuz Lake, Kazakhstan
USA	United states of America
USD	United states currency (Dollar)
UV	Ultraviolet
v	Volume
VC	Vinh Chau salt ponds, Vietnam
v/v	Volume per volume
WT	Wild type yeast strain
w/v	Weight per volume
YNB	Yeast nitrogen base culture medium
$\mu Em^{-2}S^{-1}$	Microeinstiens per meter square per second
µg	Microgram

µl	Microlitre
µm	Micrometre
µM	Micromole

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Chapter 1

Introduction, Objectives and Thesis Outline

1.1. Importance of aquaculture

According to United Nations estimates, the world population has reached 7.3 billion in 2014. New challenges arise around the world to provide sufficient and adequate food for those people. High protein foods are a necessary requirement for good health. Fish and other aquatic products are valuable sources of high quality proteins, minerals and vitamins. Moreover, fish, especially marine fish, is rich in ω-3 polyunsaturated fatty acids (n-3 PUFA), the health benefits of which are broadly recognized (Stone, 1996).

The world's consumption of seafood is on the rise. In fact, the consumption of seafood has doubled in the last thirty years and the global average fish consumption per capita per year reached 20.1 kg in 2014 (FAO, 2016). However, capture fisheries cannot deal with the growth of the world population. The controlled farming of aquatic species has expanded, diversified, intensified and technologically advanced, and its contribution to seafood production has increased significantly. The total aquaculture production has increased more than 100-fold from 0.64 million tonnes in 1950 to 73.8 million tonnes in 2014 (Fig. 1.1). Currently, aquaculture provides almost 45 % of the world's seafood and by the year 2030, aquaculture is expected to contribute at least 60 % of the world's seafood (FAO, 2016). Besides aquaculture's contribution to food security for humans, it also contributes to the world economic growth. The world's export trade of fish and fisheries products was worth approximately USD 55.75 billion in 2000, and it has continued to grow, reaching USD 160.2 billion in 2014. It is foreseen that the trend will continue to increase and that aquaculture will be the major contributor to meet the increasing demand of the growing world population for aquatic food and will be an increasingly important economic activity. Clearly, aquaculture will have a central role in the challenge to fulfil human food demand in the future.

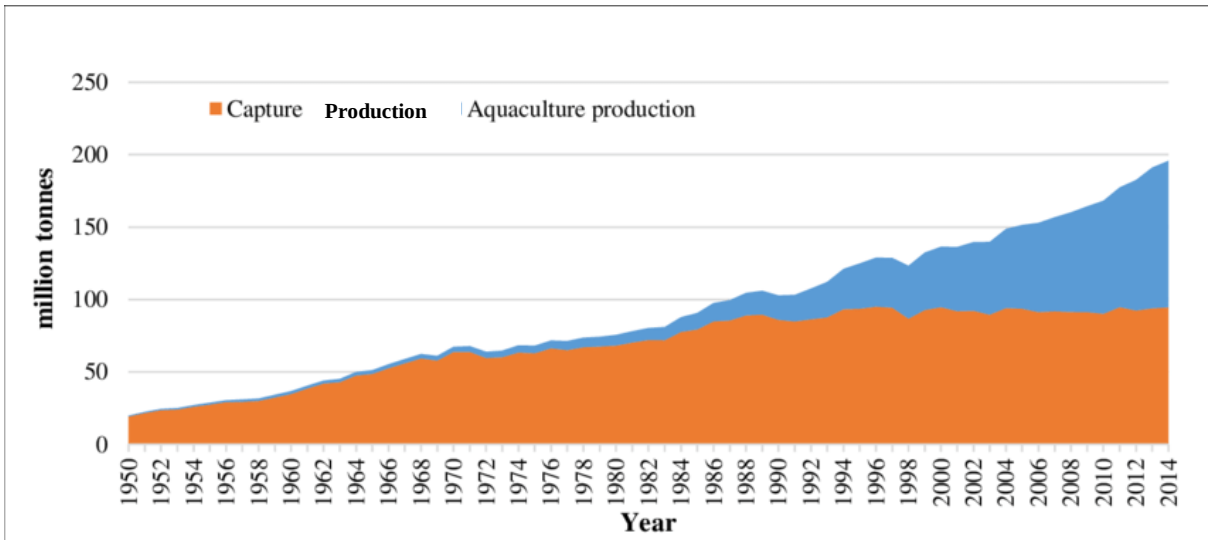

Figure 1.1: World capture fisheries and aquaculture production from 1950 to 2014 (Source: FAO, 2016).

1.2. Importance of live food and its role in start feeding aquaculture species

Live food organisms include all plants (phytoplankton) and animals (zooplankton) grazed upon by the larval stages of economically important fish and shellfish. Phytoplankton organisms are generally eaten by zooplankton. Thus, phytoplankton forms the basis of the food chain. Live food organisms are present in the water column, are constantly available to fish and shellfish larvae and are likely to stimulate a larval feeding response (David, 2003). In an aquatic ecosystem, these live food organisms constitute the most valuable food source for larval stages.

As early as the 1970's it was realized that suitable live food is a problem in larviculture (Goodwin and Hanson, 1975). Sorgeloos et al. (1987) asserted that intensive larval production of most marine fish and crustaceans was hampered by the requirement for live food, at least during the early stages of development. Within an aquaculture context, techniques for collecting or culturing the natural diet of fish and shrimp larvae are commercially unfeasible and/or technically hard to realize. On a larger and industrial scale, a readily available diet thus has to be selected according to the following

prerequisites: it can easily be accepted and digested by the larvae and has a high nutritional quality. The successes booked so far in the hatchery production of fish fingerlings and shrimp postlarvae for stocking in grow-out production systems have been largely dependent on the availability of suitable live food for feeding the larval stages (Lim et al., 2003). The digestive tract of fish larvae of many species lack important digestive enzymes and on first feeding, they rely on a food source that has to be easily digestible (New, 1998). Moreover, this food source should contain enzymes that allow auto-digestion of food particles and must have all the nutrients that are required by the larvae in terms of proteins, lipids, carbohydrates, vitamins, minerals, amino acids and fatty acids. So providing appropriate live food at proper time plays a major role in achieving maximum growth and survival of larval fish and shellfish and therefore the nutritional components of natural foods must be identified and quantified. Advances in live food enrichment techniques have further helped to boost the importance and potential of live food organisms in raising larvae of aquatic species. It is not expected that live food can entirely be replaced by manufactured feeds in the foreseeable future due to fundamental prey triggering in the fish larvae hunting behaviour (Nielsen et al., 2017).

An extensive list of potential organisms may meet the requirement of acceptability, digestibility and high nutritional quality. Of these organisms *Artemia* seems to meet the requirements best. Indeed, Kinne (1977) indicated that "more than 85% of the marine animals cultivated thus far have been offered *Artemia* sp. as food source, either together with other foods or, more often, as a sole diet" (Lavens and Sorgeloos, 1996; Sorgeloos et al., 2001). The ability of *Artemia* to produce cysts (*i.e.* embryos in arrested development) accounts in part for its convenience as a larval food source (Léger et al., 1986). *Artemia* cysts can be harvested from different production sites; they can be

shipped around the world and stored as a dry product, showing a remarkable shelf life. The ease and simplicity of cyst hatching (within 24 h), resulting in *Artemia* nauplii, make brine shrimp one of the most convenient, least labor-intensive live foods available for aquaculture. These nauplii have good palatability and induce a good and fast feeding response in fish and shellfish larvae. Moreover, *Artemia* nauplii have been used massively as vehicle for enrichment with selected fatty acids, vitamins, other essential nutrients (Léger et al., 1986; Camargo et al., 2005; Gomes et al., 2007) and therapeutic agents (Cook et al., 2003; Gomes et al., 2007). They have thus become commonly known as "living capsules of nutrition" (New, 1998).

1.3. *Artemia* cyst production in the primary producing countries

The consumption of *Artemia* cysts has increased exponentially, in parallel with the development of aquaculture (Dhont and Sorgeloos, 2002; Lim et al., 2002). In 1997, approximately 6000 hatcheries required over 1500 tonnes of dry cysts annually (Lavens and Sorgeloos, 2000a). More recently the global demand for *Artemia* cysts has almost doubled and annually is now in the range of about 3000 tonnes of dry *Artemia* cysts, (FAO, 2017). For many decades the Great Salt Lake (GSL), Utah, USA, produced about 90% of the world's production (Fig. 1.2) and only 10% was derived from a variety of salt lakes and salt works located in northern and central China, southern Siberia, San Francisco Bay, South Vietnam, and north-eastern Brazil (Lavens and Sorgeloos, 2000a).

Figure 1.2: Harvesting methods at Great Salt Lake, Utah, USA. (Source: https: //wildlife.utah.gov/gsl/harvest/collection.php).

However, the production of *Artemia* cysts from inland salt lakes was not constant and the demand of this product for hatcheries sometimes exceeded the supply. This prompted to increased exploitation of new locations in semi-arid areas (Vanhaecke et al., 1987; Triantaphyllidis et al., 1998), especially in continental Asia (Lavens and Sorgeloos, 2000a). As a consequence, the importance of the GSL *Artemia* resource has diminished somewhat because other regions in the world have greatly increased their production. The current percentage contribution of the GSL has decreased from 90 % of the supply to between 40 – 60 % over the last two decades (FAO, 2017). On the other hand, at the end of the previous century, the GSL ecosystem has been subjected to climatic and anthropogenic influences; this was reflected in unpredictably fluctuating and sometimes dramatically low harvested cyst quantities. The situation has returned to normal since then, with annual harvests in the order of 2000 – 3000 tonnes of finished product (Fig. 1.3).

Currently, in addition to the USA, Russia, Kazakhstan and China are the main suppliers in the world market of *Artemia* cysts. According to official statistics, in recent years Russia has produced approximately 1100 tonnes of cysts in wet weight (550 tonnes dry product) annually, harvested

from several lakes, while in Kazakhstan and Uzbekistan annual harvests, roughly estimated, were about 500 and 20 tonnes dry weight, respectively (Litvinenko et al., 2015). A substantial source of *Artemia* cysts is also China. China is not only a producer of *Artemia* cysts but according to FAO (2017), China is the primary global consumer of *Artemia* with an annual consumption of 1500 tonnes of dry cysts, of which approximately 900 tonnes were harvested in China in 2012/2013 (Litvinenko et al., 2015).

Moreover, trying to meet the demands of this product, and in addition to inland salt lakes where natural populations of *Artemia* are harvested for cysts, the technique of *Artemia* culture in ponds and saltworks has been introduced throughout the world (Vos and Tunsutapanich, 1979; De Los Santos et al., 1980; Camara and Tackaert, 1992; Brands, 1996; Hoa et al., 2007; Sultana et al., 2011; Sivagnanam et al., 2011). This is especially true for the regions of South and East Asia. In Vietnam there are decades of experience in producing *Artemia* cysts in controlled pond systems, especially in the Mekong Delta. In 2014 Vietnam produced almost 25 tonnes of dry cysts from an area of 1200 ha (Hoa and Sorgeloos, 2014) (Fig. 1.3.).

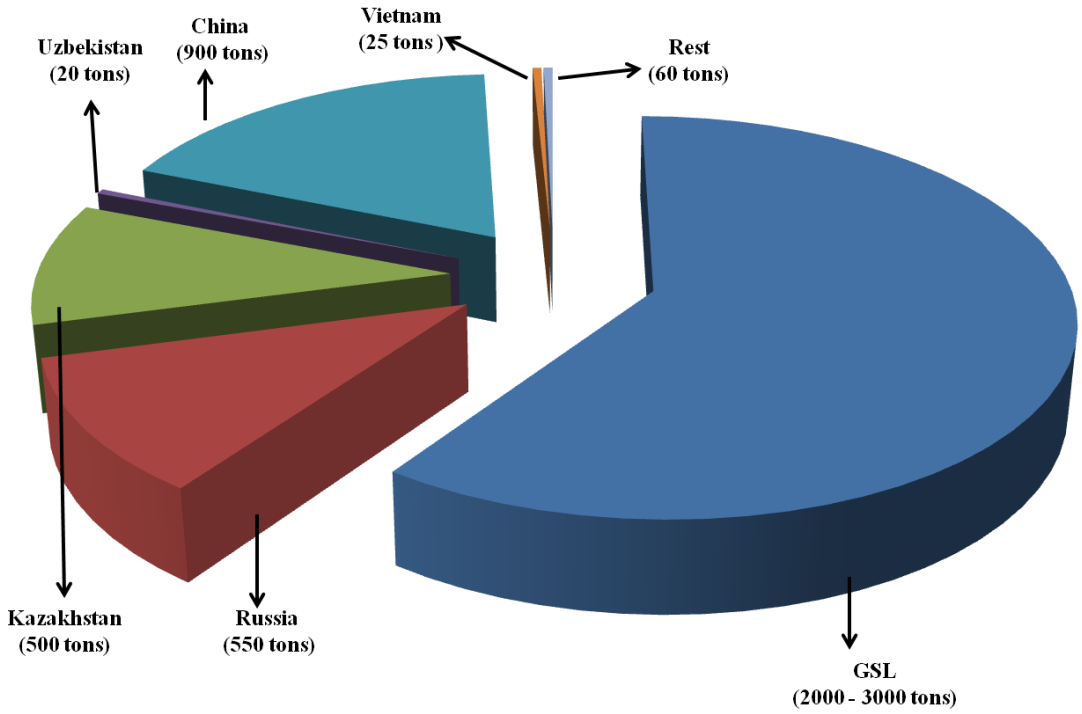

Figure 1.3: Total *Artemia* cyst production harvested from the main sources in the world (values are in dry tons) (Source of data: Litvinenko et al., 2015; Hoa and Sorgeloos, 2014).

1.4. Role of *Artemia* in aquaculture and other applications

Regardless of the vast improvements in the aquaculture feed industry there is still no artificial feed formulation available to completely substitute for the live food *Artemia*. In fact, the brine shrimp *Artemia* remains essential in most marine finfish and shellfish hatchery operations especially during the earliest life stages (Kolkovski et al., 2004). Within the aquaculture industry, shrimp hatcheries are the biggest consumers of *Artemia* cysts, utilizing about 80 to 85 % of total market availability, while the remainder is used mainly in marine fish larviculture and the ornamental fish industry (FAO, 2017). Nutritionally, newly hatched *Artemia* nauplii are high in protein and lipids, constituting an excellent food source for fish and shrimp larvae (Dhont et al., 1993). Other important features of *Artemia* nauplii are the presence of essential fatty acids (EFA) that

determines its nutritional value for larvae of various marine fishes and crustaceans (Léger et al., 1986), and also of several proteolytic enzymes, which play a significant role in the breakdown of *Artemia* in the digestive track of predator larvae, which have low levels of digestive enzymes especially at their early larval stages. Furthermore, the ability of *Artemia* to feed on suspended particles and their non-selective filter feeding characteristics allow ingestion of bioencapsulated nutrients, pigments (Sorgeloos et al., 2001), antimicrobial agents (Dixon et al., 1995), vaccines (Campbell et al., 1993) and probionts (Gatesoupe, 1994), and different techniques have been developed to enhance the nutritional profiles of nutritionally deficient *Artemia* strains. This has led to improvements in larviculture outputs, not only in survival, growth and success of fish and crustacean metamorphosis, but also by reducing the incidence of malformations while improving pigmentation and stress resistance (Van Stappen, 1996; Harzevili et al., 1998; Ringo and Birkbeck, 1999).

Besides the use of *Artemia* nauplii as live food, *Artemia* biomass (in live and frozen form) is widely used by aquarium hobbyists, fish breeders and aquaculturists (Anh et al., 2011). *Artemia* biomass is rich in protein, lipids, attractants, pigments and other active substances making it an attractive direct feed for fish or an excellent ingredient for aquafeeds, *e.g.* as a maturation trigger for shrimp broodstock and as nursery feed for marine fish, shrimp and crabs (Anh et al., 2011; FAO, 2017). In addition, it is even used for human consumption in some countries. Sun-dried *Artemia* biomass was consumed centuries ago by primitive tribes in America, Asia and Africa: "*Indians inhabiting this region used to collect large quantities of this crustacean and use it as food*" (Jansen, 1918). The Dawada-people living around Gaber-oun lake in the Idehan Ubari desert region of the Libyan Sahara (Fig. 1.4) consume dried *Artemia* flakes as "*a superb source of protein rich in β-carotene and riboflavin*" (Ghannudi and Tufail, 1978).

Figure 1.4: Gaber-oun Lake in the Idehan Ubari desert region of the Libyan Sahara (source: Best of Libya @BestOfLibya).

Additionally, *Artemia* cysts are used to grow insects within the framework of biological control of crop pests (De Clercq et al., 2005; Vandekerkhove et al., 2009). Decapsulated brine shrimp cysts can be used as a feed supplement and an economically viable alternative food source for the indoors mass production of different species of predator insects. *Artemia* is also used as a model species in evolutionary studies as the cysts, present in the habitat, can be viewed as gene banks that store the genetic memory of historical population conditions (Djamali et al., 2010; Manaffar et al., 2011). Lenormand et al. (2018) showed that *Artemia* has multiple features making it an attractive model for resurrection ecology theories, and showed in detail how cysts can be recovered from sediments to document the history and dynamics of for example biological invasions.

Apart from what is mentioned above, there are certain limitations associated with the use of *Artemia* cysts and nauplii as a live food in aquaculture. Differences in cyst size and naupliar length between populations of *Artemia* are well documented (Vanhaecke and Sorgeloos, 1980). Lavens and Sorgeloos (1996) reported that different *Artemia* cyst batches, harvested from the same lake, may present different hatching characteristics. The nutritional quality of *Artemia* may vary considerably between strains and species (Watanabe et al., 1978; Léger et al. 1986; Bengtson et al., 1991). These properties may affect the successful use of *Artemia* as live food. So a variety of cyst products with different qualitative characteristics is offered in the market. In addition, techniques for cyst harvesting and processing are sometimes not standardized which could lead to inferior quality of cysts. Overall quality of cysts may furthermore depend on features such as the season in which the cysts were produced, the food quantity and quality available to the reproducing adults, cyst diapause characteristics, levels of possibly toxic compounds. Additionally, cysts may encounter exposure to high levels of UV radiation, to extreme high or low temperatures, prolonged anoxia, high salinity or repeated cycles of hydration and severe desiccation from the moment that they are released by the female into the water, while they are suspended in the water column, or as they accumulate along the shores and become covered by sediment and debris etc. (Fig. 1.5). As *Artemia* still remains a natural commodity, diversification of resources remains an important issue along with the further rationalization of its use.

Figure 1.5: *Artemia* cyst accumulation on the Great Salt Lakes shore (left; source: https: //wildlife.utah.gov/gsl/harvest/collection.php) and in a salt pond in Thailand (right; source: Laboratory of Aquaculture & *Artemia* Reference Center)

1.5. Rationale and objectives of the study

Since aquaculture is developing rapidly in the world, the future demand for the brine shrimp *Artemia* as a live food in most marine finfish and shellfish hatchery operations is not expected to diminish at short-term, especially as no artificial feed is available to completely substitute for the live food *Artemia*, especially in terms of predator acceptance, nutritional and other characteristics. Moreover the production of *Artemia* cysts from inland salt lakes is not constant and the demand of this product for hatcheries sometimes exceeds the supply. Consequently, the reliable supply of sufficient amounts of good quality cysts remains a priority. Techniques applied for harvesting, processing and storage may negatively affect cyst quality, and hatching techniques may not be appropriate. An important impact of the manipulations during processing and storing can be related to effects of dehydration or combined dehydration and rehydration. For diapausing cysts, hydration/dehydration (H/D) may also interfere with the diapause induction/termination process,

but for quiescent cysts, uncontrolled dehydration and hydration may result in a significant drop of viability of embryos. On the other hand, it has also been reported that feeding the brine shrimp larvae with selected feeds and/or supplements may improve their viability when exposed to stress, and may provide protection against diseases and challenge by pathogens (Marques et al., 2006a).

In this context this thesis consists of two main parts. In the **first part** of our thesis, the objective was thus to evaluate how environmental stresses (*i.e.* hydration/dehydration cycles) in nature or during manipulation (*i.e.* harvesting, processing and storing) (Fig. 1.6 A) may affect the quality of cysts and the emerging nauplii used in larviculture operations. Moreover, we tried to find out to what extent exposure of the cysts to this environmental stress would deteriorate the fitness of the nauplii when exposed to abiotic and biotic stressors (Fig. 1.6 B), and if their stress resistance could be enhanced by adequate feeding (Fig. 1.6. C). This is important when using *Artemia* in aquaculture hatcheries or when using it as a model organism in aquaculture research.

In **a second part** of our thesis, we focused on optimizing the use of *Artemia* cysts by investigating their hatching biology and to stimulate the development of protocols that break diapause, more specifically the role of light in the hatching process. According to literature information, light may play a role in diapause termination, but also in the hatching process of post-diapausing cysts. Though information is available on the effect of different wavelengths and light intensities, there is only scattered information on the optimal timing and duration of light exposure during the hatching process (Fig. 1.6 D). Moreover, most studies have only been using *Artemia franciscana* so far. Finally, it is not known to what extent the light trigger may interfere or act in complementarity with the chemical termination of diapause through reactive oxygen, such as when using hydrogen peroxide (H_2O_2) or nitric oxide (NO) (Fig. 1.6. E).

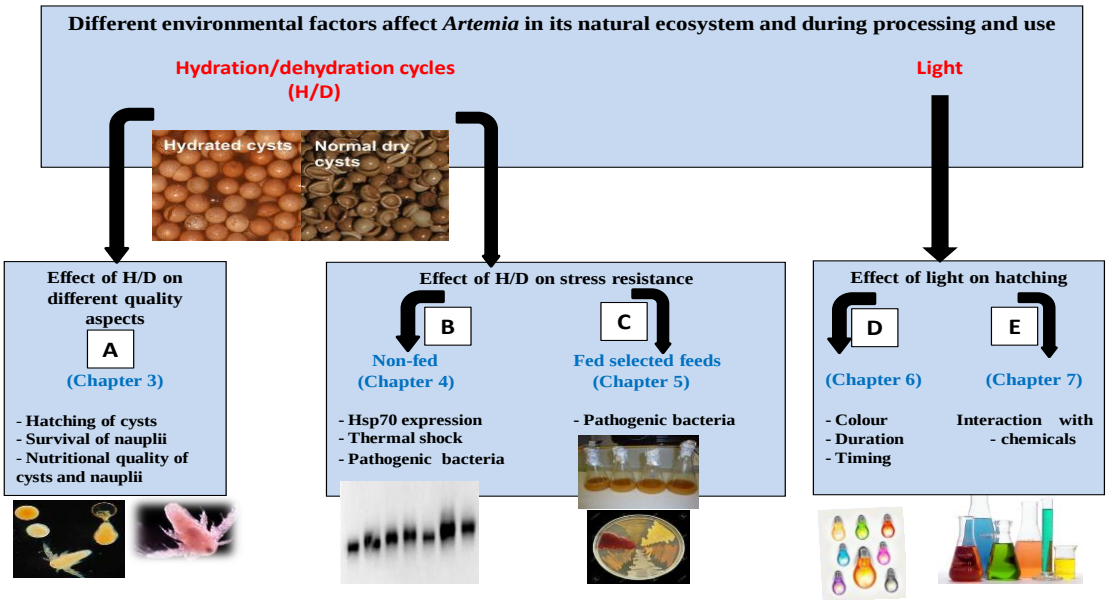

Figure 1.6: Schematic overview of the PhD thesis.

Therefore, firstly two *A. franciscana* strains which are of prime importance for global cyst supply were subjected to different treatments, each including one or more H/D steps. Assuming that progressive H/D exposure would result in progressive quality deterioration of the cysts, the quality of the resulting embryos and nauplii was assessed using practical criteria relevant for their use in aquaculture, as described in **Chapter 3**, *i.e.* cyst hatchability, naupliar longevity, cyst and naupliar energy content and naupliar fatty acid and vitamin C content.

In a subsequent study **(Chapter 4)**, we investigated, using the gnotobiotic *Artemia* rearing system, the stress response of nauplii emerging from cysts previously exposed to the H/D cycles used in Chapter 3, assuming that exposure of the cysts to H/D cycles affects the stress response of embryos and also would make the resulting nauplii less stress-resistant. Nauplii were exposed to an abiotic

(lethal heat shock) or to a biotic stressor (the pathogen *Vibrio campbellii*) and their survival was monitored. Moreover, the Hsp70 levels in the hydrated/dehydrated cysts and in the emerged nauplii were determined in order to establish a link between the levels of this heat shock protein and the stress response.

In **Chapter 5** we finally investigated if the resistance of the nauplii, emerged from cysts exposed to the same H/D cycles as used in the previous chapters, and challenged with the pathogen *Vibrio campbellii*, could be enhanced by feeding them with different food sources (baker's yeast and bacteria), by measuring their survival and growth post-challenge.

In the last two chapters three strains of *Artemia* cysts were used in the experiments: one strain of *Artemia franciscana* and two strains of parthenogenetic *Artemia* that were in different state of diapause. The effect of different aspects of illumination on the hatching of cysts was investigated. Different light parameters (colour, timing and duration of light exposure) were used during the hatching incubation process, as described in **Chapter 6**, for the three strains mentioned.

Next the different light colors, as used in Chapter 6, were used in combination with two different chemicals, *i.e.* H_2O_2 and NO in the following experiment **(Chapter 7)**, in which we aimed to investigate whether there is any interaction between the effect of light and the effect of these chemicals in the *Artemia* hatching process. Also possible strain-specific differences as a response to different light parameters and chemicals were assessed.

In **Chapter 8**, the results produced in the various chapters are discussed. The main conclusions of the overall work and the future perspectives are given.

Chapter 2

Literature Review

2.1. *Artemia* taxonomy

Taxonomically the genus *Artemia* can be classified as follows (Martin and Davis, 2001)

Phylum: Arthropoda

Class: Crustacea

Subclass: Branchiopoda

Order: Anostraca

Family: Artemiidae

Genus: *Artemia* Leach, 1819

The genus *Artemia* is composed of parthenogenetic strains (di-, tri-, tetra- and pentaploid all-female populations) and bisexual species (populations with both males and females) found in Europe, Africa, Asia, and Australia: *Artemia salina*, *Artemia monica*, *Artemia franciscana*, *Artemia persimilis*, *Artemia tibetiana*, *Artemia urmiana*, *Artemia sinica* and *Artemia* sp. from Kazakhstan (Triantaphyllidis *et al.*, 1998).

2.2. *Artemia* biology and life cycle

The brine shrimp *Artemia* is a primitive arthropod which typically inhabits harsh hypersaline environments where other organisms cannot survive. *Artemia* can reproduce in two ways (Fig. 2.1). If living conditions are favourable, the fertilized eggs in the brood pouch of the female develop into free-swimming *Artemia* nauplii (ovoviviparous reproduction). If living conditions deteriorate (*i.e.* oxygen stress, extreme temperatures, high salinities etc…) *Artemia* has the ability

to produce dormant embryos (enveloped in a shell or chorion) about 200 – 300 µm in size, known as cysts*, that are in a state of obligate dormancy called diapause (Lavens and Sorgeloos, 1987). The embryo is surrounded by a thick shell, which is a complex and rigid structure that not only acts as a covering for the metabolically inactive dormant embryo that can remain in the resting stage for a long time, but also protects the animal from high doses of UV rays (Van Stappen, 2002; Tanguay et al., 2004).

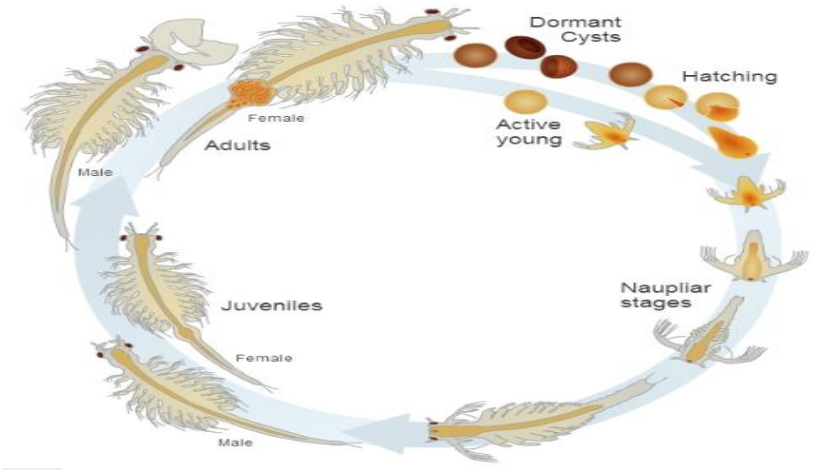

Figure 2.1: Life cycle of *Artemia* (Source: Genetic Science Learning Center).

In appropriate conditions, cysts can be produced in massive numbers, and the alveolar structure of the chorion ensures that they float on the water surface and that they may be blown to the shore by wind and wave action, where they accumulate along the shorelines (Persoone and Sorgeloos, 1980; Triantaphyllidis et al., 1998). The cysts in nature are dried by the sun. The cysts resume metabolism and further develop when hydrated. During hydration, the aerobic metabolism comprises – among

*In aquaculture, the resting eggs of *Artemia* are commonly referred to as "cysts". Although this term is biologically not correct, we will use it henceforth in this manuscript, when we are refering to the dormant *Artemia* embryo.

others – a trehalose-glycogen conversion and trehalose-glycerol conversion that ensures energy supply for respiration and hygroscopic compound accumulation for hatching, respectively (Clegg 1964; 1965; Van Stappen 1996). After 8 to 20 h hydration the cyst shell (including the outer cuticular membrane) bursts (= breaking stage) (Figure 2.2. left) and the embryo surrounded by the hatching membrane ("umbrella") becomes visible (Figure 2.2. middle). The embryo then leaves the shell completely and hangs underneath the empty shell (the hatching membrane may still be attached to the shell). Through the transparent hatching membrane one can follow the differentiation of the pre-nauplius into the instar I nauplius which starts to move its appendages. A hatching enzyme is then secreted in the head region of the nauplius, weakens the hatching membrane, and shortly thereafter the hatching membrane breaks open (= hatching) and the free-swimming larva (head first) is born (Figure 2.2. right).

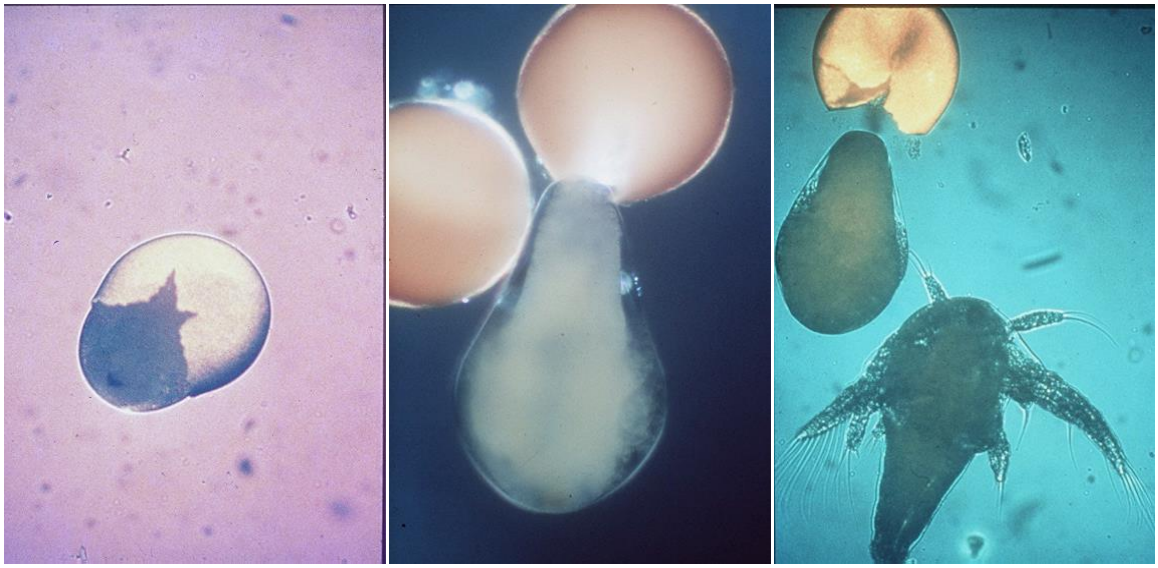

Figure 2.2: Early stages of *Artemia*: (left) breaking stage, (middle) umbrella and (right) umbrella + instar I (Source: Laboratory of Aquaculture & *Artemia* Reference Center, Ghent University).

This first larval stage with a length of about 400 – 500 µm does not feed, but relies on its energy store in the yolk, which gives this stage of *Artemia* a brownish-orange color. After a few hours,

the larva molts into the instar II stage in which the mouth opens and food uptake starts through filter feeding. The larva undergoes 15 molts until the adult stage in about 2 weeks depending on the culture conditions (Clegg and Conte, 1980; Sorgeloos, 1980). The adult has a body length of 8 – 10 mm with two lateral eyes, a linear digestive tract and 11 pairs of thoracopods. The adult female *Artemia* is recognized by the presence of the brood pouch or uterus between cephalothorax and abdomen. In the uterus embryos develop into cysts or nauplii during reproduction cycles (Sorgeloos et al., 1986). The adult male possesses a paired penis in the posterior part of the trunk and muscular graspers in the head region (Figure 2.3.).

After harvesting and processing, the cysts are available as storable "off the shelf" and "on demand" live food. The free-swimming nauplii, released after 24 h incubation in seawater, can be given directly as nutritious live food to the larvae of a variety of aquatic organisms. Its availability and the possibility to store cysts is the reason why *Artemia* has, to a great extent, been designated a convenient, suitable and excellent larval food source in aquaculture (Lavens and Sorgeloos, 2000). In addition the small size of the newly hatched nauplii is convenient for the small mouth of the fragile and not fully developed fish larvae (Lavens and Sorgeloos, 1996).

Figure 2.3: Adult female (top) and male (below) of *Artemia* (Source: Genetic Science Learning Center).

2.3. *Artemia* ecology

The ecology of the brine shrimp *Artemia* has been extensively studied. Populations of *Artemia* are typical inhabitants of hypersaline environments, such as inland salt lakes, coastal lagoons and solar salt works, distributed all over the world, scattered throughout the tropical, subtropical and temperate zones (Triantaphyllidis et al., 1998). *Artemia* can be found in a great variety of habitats in terms of water chemistry (Lenz, 1987; Bowen et al., 1988), altitude (Triantaphyllidis et al., 1998; Van Stappen, 2002) and climatic conditions, from humid-subhumid to arid areas (Vanhaecke et al., 1987). The most recent inventory of global *Artemia* zoogeography (Van Stappen, 2002) lists about 600 sites (Fig. 2.4). The distribution of *Artemia* is discontinuous: not all highly saline biotopes are populated with *Artemia*. Although brine shrimp thrive very well in natural seawater, they cannot migrate from one saline biotope to another via the seas, as they depend on their physiological adaptations to high salinity to avoid predation and competition with other filter feeders. These *Artemia* environments are characterized by ecological communities with low species diversity and simple trophic structures (Lenz, 1987; Lenz and Browne, 1991). From an ecological point of view, *Artemia* is a keystone taxon in hypersaline food webs, where it constitutes the dominant or exclusive macrozooplankton. It is the main prey for aquatic birds (Sánchez et al., 2006) and the main consumer of phytoplankton. These environments were generally considered in the past as habitats having overall ecological characteristics largely similar all over the world (Persoone and Sorgeloos, 1980; Lenz, 1987; Lenz and Browne, 1991). This underestimation of the diversity in physical, chemical and biotic characteristics of *Artemia* habitats may have been inspired by a lack of interest because, as Williams (1991) appropriately points out, salt lakes are generally considered less ‘useful’ than freshwater bodies.

The common feature of all *Artemia* biotopes is their high salinity, and salinity is without any doubt the predominant abiotic factor determining the presence or absence of *Artemia* and consequently limiting its geographical distribution. Other variables (temperature, light intensity, primary food production) may have an influence on the quantitative aspects of the *Artemia* population, or may cause only a temporary absence of brine shrimp. *Artemia* strains have been the subject of various salinity studies, which have demonstrated different physiological tolerances to salinities, specific ions and ionic ratios for different populations, although a lot depends on the salinity range studied (Cole and Brown, 1967; Persoone and Sorgeloos, 1980; Bowen et al., 1985, 1988; D'Agostino and Provasoli, 1986; Triantaphyllidis et al., 1995; Abatzopoulos et al., 2003; Agh et al., 2008).

Irrespective of the ambient salinity, *Artemia* can withstand environments in which the ratio of the major anions and cations may be totally different from that in seawater (Persoone and Sorgeloos, 1980). The ionic composition of the habitat of *Artemia* can result in ecological isolation of particular strains (Bowen et al., 1985; 1988). This striking physiological adaptation to such extreme chemical habitats brought Cole and Brown (1967) to the conclusion that 'the ionic composition of the waters inhabited by *Artemia* varies more than that of any other aquatic metazoan'.

Also the effect of temperature on the distribution of *Artemia* has been the subject of numerous studies (Vanhaecke et al., 1984; Lenz, 1987; Browne et al., 1988; Vanhaecke and Sorgeloos, 1989; Abatzopoulos et al., 2003). No *Artemia* is found in areas where year-round low temperatures exclude its development (Persoone and Sorgeloos, 1980), but a lot of strains are found in the continental areas of North and South America and Asia with extremely cold winter temperatures, as long as high summer temperatures allow cyst hatching and subsequent colonization of the environment. According to Lenz (1987), two critical factors determine *Artemia* population

dynamics: a) whether habitat conditions allow animals to survive throughout the year or not and b) whether the seasonality of the environment is predictable or not. The genus *Artemia* has diversified into environments ranging from permanent to highly ephemeral, from seasonal to aseasonal and from predictable to unpredictable (Lenz, 1987). The large temperate lakes like Great Salt Lake and salt lakes in the Russian Federation are examples of seasonal habitats. Here, the season is primarily determined by the temperature cycle while the salinity remains constant. Therefore, animals are adapted to a rapid oviparity to ensure survival through the unfavourable cold months (Lenz, 1987). In this way, a reproductive adaptation comes to play a role when survival of the species is in danger. On the other hand, permanent and relatively aseasonal habitats, for example large permanent coastal saltworks and ponds, promote ovoviviparity.

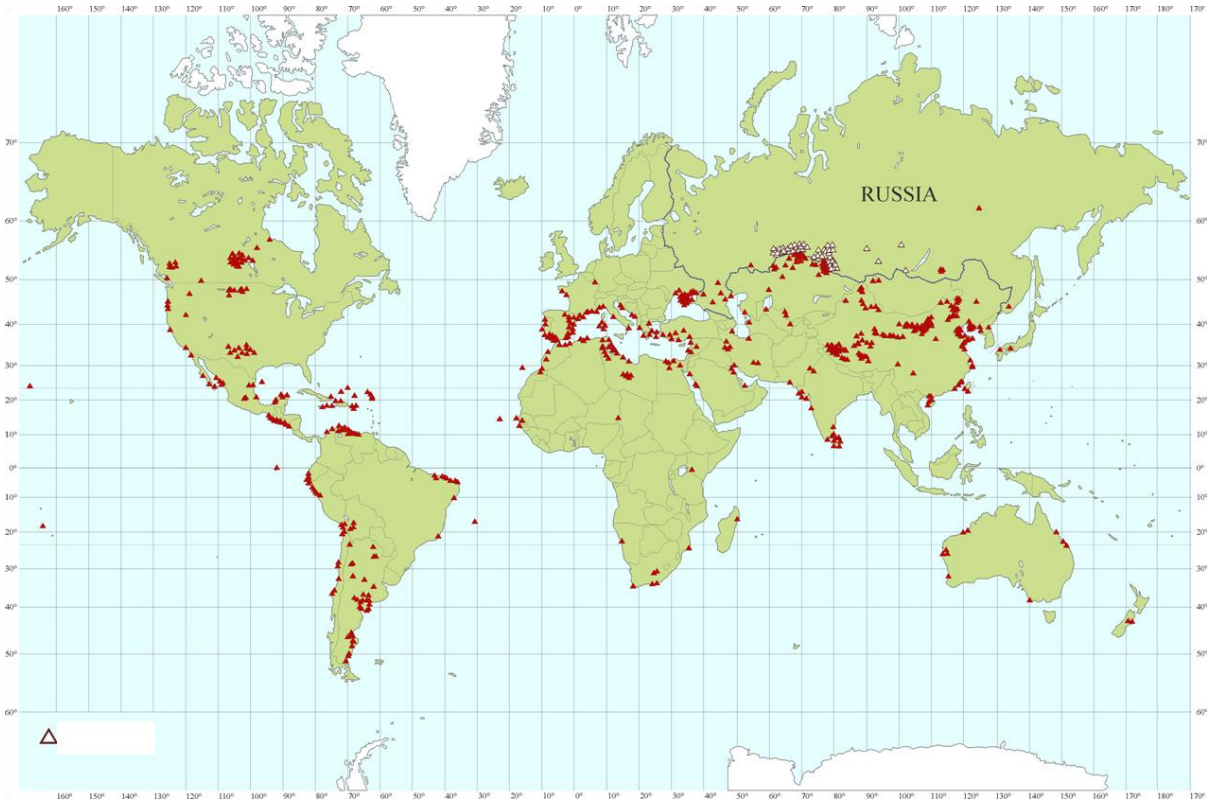

Figure 2.4: ▲ = the world distribution of *Artemia*. △ = new sites of *Artemia* distribution, described by Litvinenko et al., 2015.

2.4. Impact of natural and anthropogenic factors on *Artemia* cyst supply

In their natural environment, *Artemia* survive harsh environmental conditions where other animals would die. However, different abiotic and biotic factors may affect the reproductive characteristics of *Artemia*, such as maturation, size and number of broods, length of reproductive period (Wear et al., 1986). Increased salinity for example may cause a delay in reproduction and reduced fecundity, and thus decreasing cyst production (Wear et al., 1986). Unstable supply and variations in the yield of dry processed cysts annually are thus caused by climatic events such as fluctuations in temperature and precipitation. Over the past 10 years, annual yields of Great Salt Lake, for example, have been fluctuating between 6,000 and 15,000 tonnes of raw material, although human factors, such as reduced standing stock due to overharvesting, may also have contributed to these fluctuations. Similarly fluctuations and variation in cyst catches in different lakes in Russia due to dryness and salinity change have been recorded (Litvinenko et al., 2015).

In terms of quality of cyst yields, also the impact of contaminants such as pesticides and heavy metals on quality of the *Artemia* cyst harvest has been investigated. These heavy metals enter the lake through rain (atmospheric deposition) and river inflows. Since there is no outlet, heavy metals, often in the form of inorganic soluble salts, accumulate and they could become toxic to life forms. More recent studies (Great Salt Lake Ecosystem Program, 2017) show that the chemistry of Great Salt Lake is converting the mercury and thus makes it available for absorption into algae and microorganisms. Brine shrimp feeding on algae thus incorporate heavy metals into their fatty acids up to levels that can become toxic for *Artemia* itself and for other animals higher up in the food chain. Another kind of anthropogenic disturbance, widely occurring in *Artemia* habitats, is eutrophication. The Great Salt Lake ecosystem provides an interesting situation to study biological responses to nutrient input. The lake receives high levels of industrial, urban, mining and

agricultural discharge. The construction of a railroad causeway in 1959 divided the lake into two water bodies, affecting the biogeochemistry and distribution of nutrients (Naftz et al., 2008). Reconstruction of changes in sediment and water quality of Great Salt Lake from the early 1700's to 1998 showed that the period from 1979 to 1998 was the most contaminated (Naftz et al., 2008). This anthropogenic disturbance results in lower variety and quantity of food available (*i.e.* algae), which in turn affects the success of the *Artemia* populations and the yields of cysts harvested from the lake.

Finally, one of the main components of anthropogenic global change is the introduction of invasive species (Vitousek et al., 1996). Undeliberately, through the use of its cysts in aquaculture, but also intentionally through integrated *Artemia*-cum-salt production (Sorgeloos et al., 2001), cysts of *A. franciscana* have been introduced in many hypersaline systems, rapidly replacing native *Artemia* species worldwide (Amat et al., 2005, 2007). This contributes to the complexity of the species status of cyst product originating from these areas, which may, such as in the case of *Artemia* cyst product from Bohai Bay, China, consist of variable mixtures of parthenogenetic strains (the autochthonous coastal populations) and the allochthonous *A. franciscana* (Van Stappen et al., 2009). Due to its high reproductive capacity in a broad range of ambient conditions, *A. franciscana* outcompetes parthenogenetic *Artemia* and *A. salina* within two or three generations under laboratory conditions (Abatzopoulos et al., 2002). In nature, native populations may thus come under severe threat from competition with invasive ones, to the point of competitive exclusion of the former by the latter. Available data suggest that once *A. franciscana* is detected among native *Artemia* in existing populations, native *Artemia* may disappear within a few years (Amat et al., 2005).

2.5. Diapause as life strategy in *Artemia* and other crustaceans

Many aquatic crustacean taxa, such as Branchiopoda, Ostracoda or Copepoda, but also other invertebrates such as rotifers, depend on the production of long-lived dormant stages which allow them to persist under unfavourable conditions (drought, high temperatures, extreme salinities, predation or food scarcity), especially in highly fluctuating environments (Hand et al., 2016). These dormant stages form egg banks that can be stored in the environment, allowing for an escape strategy in the form of dispersal through time (Venable and Lawlor, 1980). Diapause is a specific type of dormancy that is genetically programmed and triggered by endogenous physiological factors in response to environmental cues (Denlinger, 2002; Koštál, 2006; Denlinger et al., 2011). Typically diapause is entered prior to the onset of adverse environmental conditions, and is characterized by ontogenetic arrest for a period spanning weeks to years (Koštál, 2006). Developmental arrest may be accompanied by metabolic arrest depending on the developmental stage and the species (Denlinger, 2002; Reynolds and Hand, 2009; Hahn and Denlinger, 2011; Hand et al., 2011; Denlinger et al., 2011).

Although the formation of a dormant stage during the reproductive part of the life cycle is not unique in crustaceans, brine shrimp seem to have developed a sort of flexibility towards the variety of 'unstable' habitat they inhabit. Unlike related phyllopods, *Artemia* females easily switch from cyst production (oviparity) to live nauplii birth (ovoviviparity), resulting in a fast increase of the population when environmental conditions are optimal. Moreover, there is no sexual control over these modes of reproduction, in contrast with for example, most cladocerans or rotifers where only fertilized, mictic females produce resting eggs. In fact, this flexible life-history strategy may also explain why some strains inhabiting relatively stable biotopes appear to have lost the ability to produce dormant cysts, *i.e.* this second mode of reproduction has no adaptive value anymore

(Lenz, 1987; Lenz and Dana, 1987). Diapause plays a vital role in maintaining the viability of *Artemia* embryos for an extended period after their release from the female (Denlinger, 2002). Diapausing *Artemia* embryos can also be protected against damage from free oxygen radicals due to down-regulation of mitochondrial activity during diapause (Clegg et al., 1996; Reynolds and Hand, 2004).

In the post-diapause type of developmental arrest in *Artemia* (see section 2.6.1), cysts are in a state of quiescence. This refers to the environmental (exogenous) control of metabolism and development, in which for example extremes of temperature, oxygen, and desiccation induce a state of retarded development; further embryogenesis will only resume when the environmental conditions become favorable (Crowe et al., 1987). Delayed hatching of resting eggs was also, for instance, demonstrated in freshwater crustaceans in relation to biotic cues such as kairomones signaling a predation threat by fish in permanent waters (Lass et al., 2005) but also by turbellarians (Brendonck et al., 2002; De Roeck et al., 2005) and by amphibians (Blaustein, 1997; Spencer and Blaustein, 2001) in temporary pools.

So from an ecological point of view, the most appropriate strategy in biotopes with more or less predictable cyclic environmental stresses would be dormancy, whereas quiescence would be optimal in non-cyclic circumstances (Belk and Cole, 1975). By the production of diapausing eggs the organisms may anticipate to sub-optimal biotope conditions. Dormancy may also be relevant as a life-history strategy, *i.e.* by synchronizing the life cycles to the variation that occur in the habitat. Endogenous control over metabolism and development ensures a synchronous hatching, resulting in a fast start and consequent development of the population shortly after the re-establishment of favourable environmental conditions. It is this synchrony that allows effective colonization in temporal biotopes.

Diapause has also a very important role in the dispersal of aquatic invertebrates. The ability to develop diapausing resting stages facilitates species survival during movements across geographical barriers and under extreme conditions (Van Stappen, 1996; Saygi, 2003). Resting eggs survive passive transportation by wind and waterfowl, and can be used for active transportation by men into new suitable biotopes (Persoone and Sorgeloos, 1980).

2.6. The *Artemia* cyst hatching process

2.6.1. Artemia diapause deactivation

In principle, *Artemia* embryos released as cysts in the medium are in diapause and will not resume development even under favourable conditions unless the diapause process is interrupted (Drinkwater and Crowe, 1987). Release from diapause is called 'activation' and usually requires transient exposure to a specific environmental stimulus (Drinkwater and Crowe, 1987). Upon the interruption of diapause, cysts enter the stage of "quiescence", meaning that metabolic activity can be resumed at the moment they are brought in favourable hatching conditions, eventually resulting in hatching. In the quiescent phase the metabolic arrest is uniquely dependent on external factors (Dhont and Van Stappen, 2003).

For the user of *Artemia* cysts several techniques have proven successful in terminating diapause. However, the sensitivity of *Artemia* cysts to these techniques shows strain- or even batch-specificity, hence the difficulty to predict the effect on hatching outcome. In many cases the removal of cyst water is an efficient way to terminate the state of diapause. This can be achieved by drying the cysts at temperatures not exceeding 35°C to 40°C or by suspending the cysts in a saturated NaCl brine solution (300 g L^{-1}). As some form of dehydration can be considered part of most processing and/or storage procedures, diapause termination often does not require any

particular extra manipulation. Nevertheless, with some strains of *Artemia* cysts, the usual cyst processing techniques do not yield a sufficiently high hatching quality, indicating that a more specific diapause deactivation method is necessary. Exposure to low temperatures (hibernation), decapsulation or incubation in a hydrogen peroxide solution (H_2O_2) have proven to be successful for diapause deactivation, when applied with specific sources of *Artemia* cysts (Van Stappen, 1996). Again, the sensitivity of a strain (or batch) to a specific H_2O_2 solution is difficult to predict. Overdosing results in reduced or absence of hatching, presumably as a result of toxicity of the chemical (Dhont and Van Stappen, 2003).

Also nitric oxide (NO) has proven to affect the state of diapause at much lower concentrations compared to H_2O_2, though more cysts went out of diapause when using H_2O_2 (Robbins et al., 2010). A different internal pH exists between diapausing and quiescent cysts, and depression of internal pH could lead to diapause breaking (Crowe et al., 1987; in Lavens and Sorgeloos, 1987). However, these authors also found that there might be two separate compartments in dormant cysts that have a different internal pH, leading to the conclusion that breaking *Artemia* cyst diapause is a complex process that requires a lot more understanding.

2.6.2. The trehalose-glycerol regulatory system

Clegg (1964) suggested an important role for the carbohydrate metabolism in the cyst's emergence process. About 98 % of the total carbohydrate of the dormant cysts, excluding chitin, consists of the disaccharide trehalose, glycerol, and a polysaccharide similar to glycogen (Dutrieu, 1960; Clegg, 1962; Carpenter and Hand, 1986). These three carbon compounds are of primordial importance. During the process of embryos entering into a cryptobiotic state, trehalose accumulates in the dormant cysts at the expense of glycogen (Clegg, 1965). The presence of

trehalose prevents the denaturation of proteins and retains cellular integrity in the cysts (Jain and Roy, 2008) as it is particularly effective in stabilizing dry membranes, phospholipid bilayers and proteins (Crowe et al., 1987). It is not maternally derived but is synthesized by the embryo itself (Clegg, 1965) and accumulates up to 15 % of the total dry weight of a dormant cyst which weighs about 2.5 µg (Clegg, 1962). In addition, this compound not only enables dormant embryos to survive stress conditions, it also serves as an energy supply for embryonic development and leads to hatching of the embryos from the cysts under suitable environmental conditions by the formation of glycogen and glycerol, respectively (Clegg, 1964; Cam et al., 2009; Yang et al., 2013).

Glycogen and glycerol are two other forms of carbon present in dormant cysts. Clegg (1962) reported that the glycogen remaining after conversion into trehalose represents about 1 – 2.5 % on cyst dry weight. Glycerol is suggested to serve as a cryoprotectant to prevent low temperature damage to the embryos (Cheng et al., 2014). It is present in dormant cysts in small quantities of approximately 2 – 5 % on dry weight (Clegg, 1962). Through its hygroscopic properties, it enables the embryo to develop and emerge successfully over a wide range of external osmotic pressures (see further).

Encysted embryos of the brine shrimp *Artemia* may be reversibly rehydrated and dehydrated (Clegg, 1967). Dry cysts are very hygroscopic and take up water at a fast rate, *i.e.* up to 140 % water uptake within the first hours of hydration. When dry, the embryos are completely ametabolic, but in less than 1 hour after they have been immersed in seawater they initiate metabolic activity (Emerson, 1963) and macromolecular synthesis (Finamore and Clegg, 1969). Although the sequence and timing of developmental events within the hydrated cyst are largely unknown, it usually has been assumed that development begins when the cyst imbibes water (Finamore and

Clegg, 1969). During hydration, the aerobic metabolism in the cysts assures the conversion of the carbohydrate reserve trehalose into glycogen (as a source of energy) and glycerol (a hygroscopic compound). The gradual accumulation of free glycerol in the cyst produces a corresponding increase in the internal osmotic pressure, resulting in further water uptake by the cyst through the outer cuticular membrane. The biconcave cyst swells and becomes spherical. Consequently, the osmotic pressure inside the outer cuticular membrane builds up continuously, until a critical point is reached. At this point, an osmotic rupturing of the shell occurs (= burst of the outer cuticular membrane and the cyst shell) and all produced glycerol is released in the hatching medium. The time point of maximal glycerol release corresponds to the time point of maximal *Artemia* cyst breaking (Thai, 2015). Clegg (1964) found that upon breaking 3.1 mg of glycerol-C was released into the medium per gram dry cysts. Higher values were reported by Thai (2015), *i.e.* in the range 18.3 – 28.5 mg C g^{-1} cysts, who also found that these values increased with increasing salinity (from 5 to 35 g L^{-1}) of the hatching medium. This supports the concept that the hygroscopic glycerol is accumulated in the cysts, as a function of ambient salinity, required for hatching (Van Stappen, 1996).

During the cyst development, external osmotic pressure thus has a major effect on the rates of development and emergence, on respiration, and on changes in glycogen, glycerol, and trehalose concentrations. In addition, the oxygen consumption of cysts decreases with increased osmotic pressure within the medium (Clegg, 1964). The complete oxidation of trehalose is the major, and perhaps the only, source of energy for development (Clegg, 1964). It can be expected that anything which sufficiently decreases the supply of energy will also slow down development and, therefore, prolonge the time required for emergence to occur (Clegg, 1964).

After breaking of the cyst shell, the embryo is in direct contact with the external medium through the hatching membrane. An efficient ionic osmoregulatory system is now in effect, which can cope with a big range of salinities, and the embryo differentiates into a moving nauplius larva. A hatching enzyme, secreted in the head region of the nauplius, weakens the hatching membrane and enables the nauplius to liberate itself into the hatching medium (Lavens et al., 1986b).

Partial dehydration or rehydration for few minutes resulting in water levels in the range 30 – 65 % H_2O will initiate metabolic activities which may critically reduce the energy contents down to levels which are insufficient to reach the state of emergence when later incubated in seawater under optimal hatching conditions. Clegg (1978; 1986) hypothesized that, as hydration levels fall below 0.60 g H_2O g^{-1} dry weight, metabolic pathways are disconnected, resulting in a restricted metabolism that does not permit hatching of the cyst. Hatching quality in stored cysts is also slowly decreasing when the cysts contain water levels in the range 10 – 35 % H_2O. Clegg and Cavagnaro (1976) detected indications of enzyme activity and a serious drop in the ATP concentration in this hydration range. These processes may be retarded when the cysts are stored at freezing temperatures (Vanhaecke and Sorgeloos, 1982).

2.6.3. Environmental factors influencing Artemia hatching success

Artemia hatching can be influenced by maternal factors (the food quantity received by the parent *Artemia* will affect its offspring hatchability) (Lavens et al., 1986a) and by environmental factors (Versichele and Sorgeloos, 1980; Dana and Lenz, 1986), thus having optimum hatching conditions is extremely important within an aquaculture context. The cysts handling process (harvesting, cleaning, drying, and storing) may have an impact on the hatchability as partial dehydration or rehydration in quiescent cysts will initiate metabolic activity, critically reducing the energy

content, making it unable to hatch even when incubated in optimum conditions (Lavens and Sorgeloos, 1987).

Salinity plays a role in hatching; if it is too low or too high it will negatively affect the hatching percentage. The optimal salinity varies among the different strains of *Artemia*, but generally *Artemia* can hatch well in a salinity range between 15 g L^{-1} and 35 g L^{-1} (Sorgeloos et al., 2001). Upon cyst incubation, salinity interferes with the glycerol amount needed to break the shell. Cysts incubated at low salinity level will have faster hatching because they need less time to reach the required intra-cystic osmotic pressure while high salinity levels will leave the nauplii with less energy reserve left (Lavens and Sorgeloos, 1987).

Temperature is known to be one of the important abiotic parameters for development of aquatic organisms. Temperature relates directly with cyst hatchability and hatching rate, nauplii molting and development rate as well as with reproductive capacity (Vanhaecke and Sorgeloos, 1989). Most strains cannot survive temperatures under 6°C and above 35°C but this is strain-specific (Browne et al., 1988). The optimal temperature for the hatching of most *Artemia* strains is in the range 23 – 30 °C (Van Stappen, 1996). Other environmental factors of importance are oxygen levels and pH: the aeration intensity must be sufficient to maintain oxygen levels above 2 mg L^{-1}, preferentially 5 mg L^{-1}, and optimum hatching is acquired at slightly alkaline conditions at a pH of 8 – 8.5. An optimum pH is needed for the hatching enzyme to digest the cuticular membrane, enabling the release of free swimming nauplii (Lavens and Sorgeloos, 1987).

Finally, light exposure has been identified as one of the prerequisites to initiate hatching in eggs of many branchiopod crustacean groups (Pancella and Stross, 1963; Bishop, 1967; Hempel-Zawitkowska, 1970; Takahashi, 1975; Mitchell, 1990; Horiguchi et al., 2009; Pinceel et al., 2013), including *Artemia* (Sorgeloos, 1973). In addition, light is thought to play an essential role in

metabolic activation and dormancy termination in both plants and animals. Light can be considered in different ways. The quality of light that is available for the hatching processes is not only characterized by its intensity, but also by the wavelength, spectrum and the length of the photoperiod. Duration of light exposure has been identified as an important factor influencing hatching mechanisms. However, in nature, light duration often varies concurrently with temperature and irradiance (*i.e.* with latitude or with season), making it difficult to separate these factors. The length and sequence of light and dark phases is also an important determinant of hatching in other crustaceans, such as in cladoceran eggs (Vandekerkhove et al., 2005). In addition, the mode of *Artemia* reproduction is amongst others influenced by the photoperiod in combination with temperature (Nambu et al., 2004): short days and higher temperature favour oviparity (cyst production), whereas long days and lower temperature promote ovoviviparity (release of free-swimming nauplii).

The physiological role of light during *Artemia* hatching has not yet been entirely understood. Several studies trying to unravel the physiological processes triggered by light exposure (Van Der Linden et al., 1985; 1986; 1988; 1991) concluded that there is a correlation between light intensity and hatching percentage. The wavelength of 450 – 470 nm is the major peak for decapsulated San Francisco Bay cysts to trigger metabolism while peaks at 525 – 575 nm resulted in a maximum hatching in non-decapsulated cysts. A photoreceptor (possibly haem pigment) was assumed to mediate the light-induced hatching. These studies also identified that light can induce pH changes within the cyst, which activate trehalose catabolism and cause the resumption of metabolism and development.

The amount of light needed may vary among strains as a consequence of different chorion thickness and haematin pigment concentration, which can lower light infiltration (Vanhaecke et

al., 1981). These authors hypothesized that the effect of light is more as a diapause deactivator rather than triggering the metabolism. Darkness also promoted embryonic diapause termination of dormant *Artemia* cysts (Nambu et al., 2008; 2009).

2.7. *Artemia* as a model organism in stress response tests

2.7.1. Artemia as a model organism to study host-microbial interactions

Artemia is widely used for scientific research as a model animal for crustaceans. In comparison with target aquaculture animals, trials using *Artemia* have a much higher throughput because of the small scale of the set-up allowing a high number of replicates and/or treatments, the short production time of *Artemia* nauplii out of cysts (18 – 24 h), the short generation time of 2 – 3 weeks for the production of live offspring by adults, and the possibility to work with sterile *Artemia* nauplii (Marques et al., 2004a, b; 2005). The animal is extremely useful for studying the biology of infections or the effect of chemotherapeutic agents on diseases in crustaceans (Overton and Bland, 1981; Criado-Fornelio et al., 1989; Verschuere et al., 1999, 2000b; Marques et al., 2005). They are useful organisms for stress response studies (Clegg et al., 2000; Frankenberg et al., 2000; MacRae, 2003), feed quality analysis (Marques et al., 2004a, b) and probionts testing (Marques et al., 2005, 2006c). New feeds and supplements, *e.g.* using bacteria or yeast, or compounds thereof, are being developed to reduce stress and mortality, maintain the health of aquaculture organisms, and to stimulate the mechanisms of non-specific defense against diseases (Marques et al., 2006d). The gnotobiotic *Artemia* test system (GART), where larvae are hatched and grown in axenic conditions, has facilitated research on host-microbe interactions (Marques et al., 2006b). This system has contributed to the elucidation of several biologically-based therapeutic alternatives for potential use in aquaculture (Marques et al., 2006a; Defoirdt et al., 2006a, b; Soltanian et al., 2007).

Contrary to pathogenic or opportunistic bacteria, there are also favourable **bacteria**, which provide advantages to the host as they may improve nutritional quality, confer protection, and/or enhance immune responses to stresses and pathogens resulting into enhanced survival rates, growth and improved overall health condition. Indeed, bacteria may constitute a source of essential proteins, amino acids, vitamins and active enzymes (Intriago and Jones, 1993; Gorospe et al., 1996) and can provide a probiotic effect as well (Verschuere et al., 2000a). The cell size of bacteria varies from one species to another, but in general ranges from 0.2 to more than 0.5 µm in diameter. The major component of bacterial cells is proteins (25 – 49 % of dry weight), followed by ash (4.7 – 14 %), carbohydrates (2.5 – 11 %) and lipids (2.5 – 9.0 %), illustrating the potential of bacteria as source of nutrients. However, bacteria may be deficient in polyunsaturated fatty acids (PUFAs), such as 20:5n-3 and 22:6n-3, (Brown et al., 1996). Bacteria are grazed by *Artemia* and can make up for a substantial part of their diet. The increased growth rate of consumers in case of bacteria addition is generally linked to digestive enzyme activities (Ziaei-Nejad et al., 2006). Increase of digestive enzymes may lead to enhanced digestion and absorption of food, which in turn contributes to the improved survival and growth of consumers (Douillet, 1987; Intriago and Jones, 1993; Gorospe et al., 1996).

Also **yeast** may be one of the food sources for filter-feeding zooplankton. Yeasts are aquatic and terrestrial unicellular fungi with good buoyancy in the water column. The cell size of yeasts varies according to the species, but in general ranges from 2.5 to 10.5 µm in width and 4.5 to 21.0 µm in length (Reed and Peppler, 1973). According to Waslien and Oswald (1975) yeasts are a rich source of proteins (45 % of yeast cell dry weight), and contain 4 – 7 % lipids, 26 – 36 % carbohydrates and 5 – 10 % ash. Particularly baker's yeast *Saccharomyces cerevisiae* has been used to culture *Artemia* (Coutteau et al., 1990; Marques et al., 2004a; Soltanian et al., 2007). In addition *S.*

cerevisiae is an excellent source of β-glucans and chitin together with mannoproteins (Magnelli et al., 2002), which are present in the yeast cell wall as major compounds. According to Marques et al. (2006c, d), the use of small amounts of baker's yeast and glucan particles (obtained from baker's yeast) in gnotobiotic *Artemia* is effective to overcome the pathogenicity of *Vibrio campbellii* and *Vibrio proteolyticus*. Moreover, yeast cells harbouring null mutants for enzymes involved in the early biochemical pathway for cell wall mannoproteins synthesis performed best as feed for *Artemia* (Marques et al., 2004a). Beside the prebiotic effects, β-glucans also have immuno-modulating activities and are therefore important for the current evolution in the aquafeed industry, which focuses on health promoters, higher feed efficiency and alternatives to antibiotics (Soltanian et al., 2007).

2.7.2. Heat shock proteins and their role in stress tolerance

Studies on stress resistance in *Artemia* and in other organisms often focus on the production of heat shock proteins. Heat shock proteins (Hsps), also called molecular chaperones or stress proteins, are a family of highly conserved intracellular proteins produced by organisms upon exposure to biotic and abiotic stress (Iwama et al., 1998; Roberts et al., 2010). Experimental evidence that Hsps are involved in conferring tolerance to environmental extremes is abundant in both aquatic and non-aquatic species (Iwama et al., 1998; Clegg et al., 2000). They are generally named on the basis of their function, sequence homology and molecular weight in Daltons, *e.g.* Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and several small Hsp families (Gething, 1997). Initially, heat shock protein molecules were found to be produced in response to heat shock. To date, they are known to be up-regulated by different stressors ranging from elevated temperature to chemical and physiological perturbations, *e.g.* cold, anoxia, UV radiation, hypoxia, microbial damage and

disease (Welch, 1993; Morimoto, 1998; Pockley, 2003), heavy metal exposure and osmotic stress (Parsell and Lindquist, 1993; Feder and Hoffman, 1999), nutritional deficiency and food deprivation (Cara et al., 2005).

In addition to being up-regulated during cellular stress, Hsps are also expressed constitutively in normal unstressed cells. These Hsps, referred to as constitutive chaperones or heat shock cognates (Hscs), represent 5 – 10 % of the total protein in healthy growing cells (Pockley, 2003). The constitutive expression of Hsps in cells enhances survival of organisms by protecting vital cellular functions and leads to maintenance of critical cellular processes such as correct assembly and protein folding, conformity and translocation (Iwama et al., 1998). When cells are stressed by abiotic or biotic insults, there is up-regulation of the inducible form of Hsps which can be detected in the cells at concentrations two or three times those of their constitutive counterpart (Pockley, 2003; Roberts et al., 2010). The low molecular weight Hsps are more species-specific in function and do not have a counterpart constitutive chaperone present within the non-stressed cell (Pockley, 2003; Iwama et al., 2004). As this family of proteins is induced as well by stressors other than heat, the term 'stress proteins' is also used to describe them. However, in this review, the Hsp nomenclature is used.

In this regard, *Artemia* cysts contain two abundant, low-molecular-mass proteins (De Herdt et al., 1979): one being p26, a small heat shock protein (sHsp) (Liang et al. 1997a, b) with a calculated monomeric molecular mass of 20.7 kDa (Sun et al., 2006). The second abundant cyst protein is termed artemin (Slobin, 1980) or the 19S complex (De Herdt et al., 1981), which is similar in amino acid sequence to ferritin, but artemin is enriched in cysteine and possesses an extended carboxyl-terminal tail not found in ferritin (Rasti et al., 2009). Artemin has a monomeric molecular mass of 27 kDa (Hu et al., 2011).

Both small heat shock proteins are important in stress tolerance in *Artemia*. The lack of p26 in embryos developing directly into nauplii, its abundance in cysts and its degradation as nauplii emerge from cysts, suggests that this sHsp functions during diapause and quiescence. Supporting this viewpoint is the observation that first instar nauplii hatched from cysts and containing residual p26 are more heat tolerant than nauplii that develop ovoviviparously and lack p26 (Liang and MacRae, 1999). Artemin is extremely stable, and can endure extended cyst storage, years of anoxia and cold, and cysts show reduced tolerance to desiccation and freezing after knockdown of artemin (King et al., 2014). Diapause-destined *Artemia* embryos synthesize a wealth of p26 and artemin before being released from females, yielding a large supply of molecular chaperones within cysts, which is significant for their importance in protecting proteins during stress. These chaperones are synthesized prior to diapause and quiescence, not in response to adverse environmental conditions, and they are active in resisting physiological and environmental stressors encountered by cysts (MacRae, 2016).

The stress response has been implicated in acquired tolerance or cross-tolerance, a phenomenon in which exposure to one stressor transiently increases the resistance of an organism, at the cellular and organismal levels, to a subsequent stressor of a same or different nature that would otherwise be lethal (Sanders, 1993; Iwama et al., 1999; Sung et al., 2011a). Li and Hahn (1978) were among the first researchers to document that cultured mammalian cells, preconditioned by exposure to a sublethal heat stress, acquire greater resistance to subsequent heat and chemical exposure. Studies in fish and shellfish have shown that a mild heat shock can increase the tolerance of cells to subsequent thermal challenges (winter flounder *Pleuronectes americanus,* Brown et al., 1992; *A. franciscana,* Frankenberg et al., 2000; Sung et al., 2007), chemical challenges (*P. americanus* renal

epithelia cells, Brown et al., 1992; Renfro et al., 1993), osmotic (Atlantic salmon *Salmo salar*, DuBeau et al., 1998), and acid challenges (Martin et al., 1998).

Heat shock proteins also play a role in the regulation of hormone and receptor interactions (Welch, 1993). In addition, they elicit an innate immune response against many diseases in organisms which do not have an adaptive immune system, while they assist in those organisms which do have a well-defined immune system (Srivastava, 2002; Robert, 2003). Numerous studies have demonstrated that Hsps are responsible for induced thermotolerance, and it has been reported that organisms showing induced thermotolerance also exhibit increased resistance to other forms of stress including exposure to pathogens (Nover, 1991; Spees et al., 2002). So the application of heat shock proteins and their inducer has shown potential in disease control and in protection against various abiotic stressors (Roberts et al., 2010). Recently, it is also assumed that simultaneous induction of multiple Hsps, including Hsp70, within the host could have a broad spectrum protective effect against a wide range of stressors in aquaculture (since Hsps are known to work synergistically, Nagai et al., 2010). In this regard, Hsp70 induced by certain natural compounds appears to confer resistance against a wide variety of stressors. This method has been shown to induce protective immunity in *Artemia* against vibriosis and is thus a potential tool to prevent *Vibrio* diseases in aquaculture (Baruah, 2012).

Chapter 3

Hatching and nutritional quality of *Artemia* cysts progressively deteriorates as a function of increased exposure to hydration/dehydration cycles

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Abstract

Nauplii hatching from *Artemia* cysts are crucial in larviculture nutrition. *Artemia* cysts may be exposed to repeated hydration/dehydration (H/D) cycles pre-harvesting or during processing and storage. To observe the effect of these cycles on cyst quality, *Artemia franciscana* cysts were exposed to a comprehensive set of various H/D treatments, differing in the number of cycles (1, 2 or 3) and the duration of the fresh water hydration period (2 or 4 h). Cyst quality was assessed using criteria of immediate relevance for aquaculture use, such as hatching percentage directly after H/D treatment and after - 18°C storage up to one month, longevity of axenically hatched starved nauplii, cyst and naupliar energy content, and (for the most extreme H/D treatment) cyst and naupliar fatty acid and vitamin C content.

Repeated H/D cycles resulted in significantly ($P < 0.05$) decreased cyst hatching, reduced starved naupliar longevity and individual energy content, loss in vitamin C and fatty acid content, and moreover a close correlation between these parameters as a function of progressive H/D treatments. This is of immediate relevance for aquaculture nutrition, as commercial *Artemia* cysts may have gone through an unknown sequence of H/D cycles in nature or in the processing line, which affects the nutritional quality of the nauplii used in larviculture operations.

3.1. Introduction

The brine shrimp *Artemia* (Crustacea, Anostraca) is the main zooplanktonic organism that inhabits hypersaline environments all over the world (Triantaphyllidis et al. 1998). This branchiopod has acquired extremely capable adaptive mechanisms to survive and evolve in habitats with extensive and often abrupt fluctuations in abiotic conditions such as salinity, UV irradiation, temperature, and oxygen concentration (Persoone and Sorgeloos, 1980). These mechanisms are poorly understood, although several studies have shown the ways that *Artemia* responds to varying abiotic conditions prevailing in its natural habitats (for review see Abatzopoulos et al. 2002 and references therein). To survive environmental stress *Artemia* has developed two different reproductive patterns, with females releasing either swimming larvae (nauplii) or encysted gastrulae (cysts) (MacRae, 2003). When cysts are produced, the embryo enters diapause, a reversible physiological condition during which metabolism is greatly reduced and stress tolerance is increased (Drinkwater and Clegg, 1991; Clegg, 1997; MacRae, 2003, 2005). Exposure to habitat-specific environmental stimuli, such as desiccation and/or low temperature, promotes resumption of cyst development and metabolism (Drinkwater and Crowe, 1987; Van Der Linden et al. 1988; Drinkwater and Clegg, 1991; Nambu et al. 2008).

The first use of *Artemia* nauplii, hatched from cysts, is known from the 1930s when this zooplankton organism was used as a suitable food source for fish larvae in the culture of commercially important species (Sorgeloos, 1980; Léger et al. 1986). Since then, *Artemia* has been found to be a suitable food for diverse groups of organisms of the animal kingdom, especially for a wide variety of marine and freshwater crustaceans and fishes (Sorgeloos, 1980).

Cyst hatching is determined by a variety of factors, including genetic factors, the degree of diapause termination, ambient conditions before and during harvesting, processing and storage procedures and ambient conditions during the hatching incubation process itself. One of the most effective methods for deactivating diapause in cysts of the San Francisco Bay (SFB) type *Artemia franciscana* (Kellogg 1906) in laboratory conditions is dehydration or well-controlled consecutive hydration/dehydration (H/D) cycles (Sorgeloos et al. 1976; Vanhaecke and Sorgeloos, 1982; Lavens et al. 1986a). Quiescent cysts (out of diapause) on the other hand may go through H/D cycles when being exposed to ambient conditions pre-harvest in natural habitats or during processing procedures. This may result in variable quality loss exemplified by reduced and/or delayed hatching especially after storage (Vanhaecke and Sorgeloos, 1982; Lavens and Sorgeloos, 1987). Though some H/D exposure is, to a certain extent, an almost unavoidable element in the history of any commercial cyst sample from pre-harvesting until marketing, and though the related decline in hatching can be substantial, no systematical research has been done in this respect.

This study assumed that the loss of hatching quality as a consequence of one or more H/D cycles would be proportional to the magnitude of the exposure and the duration of subsequent storage. It further assumed that this treatment would also result in deterioration of other quality characteristics relevant for the use of brine shrimp as live food in larviculture. For this purpose, cysts of two *A. franciscana* strains (Great Salt Lake, USA, and the San Francisco Bay-type Vinh Chau, Vietnam) which are of prime importance for global cyst supply, were subjected to different treatments each including one or more H/D steps in well-defined experimental conditions. The quality of the resulting embryos was assessed using practical criteria relevant for their use in aquaculture: hatching quality, longevity of starved nauplii and nutritional quality measured as energy content, HUFA and vitamin C levels of cysts and nauplii.

3.2. Materials and methods

3.2.1. Cyst samples

Experiments were performed with two strains of *A. franciscana* collected in 2007: one commercial dry sample (water content 4.5 ± 0.2 %) originating from Great Salt Lake (GSL), Utah, USA (INVE Aquaculture Belgium, Type EG (batch number: 21425), and a second sample from Vinh Chau (VC) salt fields, Vietnam (ARC code 1718), supplied by Can Tho University, Vietnam, being dehydrated and stored in saturated NaCl brine (water content 34.8 ± 1.8 %). Both samples had been stored at + 4 °C since their arrival at the Laboratory of Aquaculture & *Artemia* Reference Center. Water content of cysts was determined by drying a subsample of raw cysts in an oven for 4 h at 103 °C to a constant weight.

3.2.2. Hydration/dehydration cycles

Cysts were exposed to successive hydration/dehydration (H/D) cycles by incubating 1.6 g of cysts of each strain in a 1 liter cylindroconical glass cone containing 800 mL of medium fresh water (tap water) for the hydration step, NaCl-saturated brine (280 – 300 g L^{-1}) for the dehydration step) at 28 °C under strong aeration. A first group of three cones was set up; the cysts in the first cone were exposed to one H/D cycle (2 h hydration, 24 h dehydration), the second one to two cycles, and the third one to three (named A1, A11 and A111, respectively). In parallel, for each strain three other cones went through a similar set-up, but with each hydration period lasting for 4 h (the corresponding treatments named A2, A22 and A222). The above operation was repeated nine times for each treatment, in order to produce sufficient cyst material for the subsequent analyses, and the samples, corresponding to the same treatment out of six, were pooled for storage and use. Cyst

samples, pooled per treatment, were immediately stored in + 4 °C in NaCl-saturated brine (280 – 300 g L^{-1}) until use for any of the tests described under 3.2.4, 3.2.5, 3.2.6 and 3.2.7.

3.2.3. Determination of hatching percentage (H %)

Determination of the hatching percentage was performed in triplicate. From each of the pooled samples having gone through the H/D cycles (and a control, not exposed to H/D), a subsample of 1.6 g of cysts was incubated in triplicate in 800 mL Instant Ocean® solution of 32 ± 1 g L^{-1} in 1 liter cylindroconical glass cones under continuous illumination (2000 lux) at 28.0 ± 0.5 °C (Lavens and Sorgeloos, 1996). Aeration was provided from the bottom to keep all the cysts in suspension.

After 24 h of incubation six subsamples of 250 µL each were taken from each cone with a micropipette and placed in a small vial. Nauplii were fixed by adding a few drops of lugol solution and tap water. The nauplii as well as the umbrellae were counted under the microscope. The unhatched cysts were subsequently decapsulated by adding a few drops of NaOCl and NaOH solution to each vial (Bruggeman et al. 1980), and the orange colored embryos were counted, according to the procedure described by (Lavens and Sorgeloos, 1996). The hatching percentage was calculated as follows:

H % = N/ (N + U + E) x 100, where N = number of nauplii, U = number of umbrellae, E = number of embryos.

The mean hatching value per cone was recorded and the overall mean hatching percentage and standard deviation for the three replicate cones were calculated. H % was determined at day 0 (= immediately after the H/D treatment), and after 1 week and 1 month of storage at - 18 °C in NaCl-

saturated brine (280 – 300 g L^{-1}). The stored samples were placed at room temperature (± 22 °C) for one day before H % testing.

The hatching rate was only determined for non-treated cysts (control), by determination of the hatching percentage obtained after a hatching incubation period of 10, 12, 14, 16, 18, 20, 22, 24 and 48 h (Lavens and Sorgeloos, 1996).

3.2.4. Axenic Artemia culture

Axenic *Artemia* nauplii of each strain were obtained following decapsulation of samples of hydrated/dehydrated cysts (obtained according to the procedure described above) and subsequent hatching procedures described by Marques et al. (2004a). A subsample of a few grams of hydrated/dehydrated cysts corresponding to each H/D treatment (and a control, not exposed to H/D) were hydrated in 90 mL tap water for 1 h with strong aeration in non-axenic conditions. The recipient with the cysts was then transferred to a laminar flow hood, where decapsulation was performed using autoclaved and sterile tools. Aeration of the *Artemia* cysts was pumped through a 0.22-µm filter. Then 50 mL of cold NaOCl containing 15 % (w/v) active chlorine and 3.3 mL of 32 % (w/v) NaOH were added to the hydrated cysts. The reaction was stopped after 150 s by adding 70 mL of sterile $Na_2S_2O_3.5H_2O$ (10 mg L^{-1}). Decapsulated cysts were washed several times carefully with filtered autoclaved sea water (FASW) and collected over a 50-µm sterile sieve. A few mg of these cysts were then transferred to separate, sterile 50-mL falcon tubes (four replicates per H/D treatment) containing 30 mL of FASW and capped. For hatching incubation, the tubes were placed on a rotor at 4 cycles/min to prevent clogging and sedimentation of the cysts. Cysts were kept at 28.0 ± 0.5 °C and exposed to constant incandescent light (2000 lux). After 18 – 20 h, 20 hatched nauplii were picked and transferred to new sterile 50-mL falcon tubes containing 30

mL of FASW, which were mounted on the rotor and incubation was continued. After 12, 24, 36, and 48 h, during which the larvae were not fed, swimming larvae were counted and survival percentage of the four replicates was calculated, as described by Baruah et al. (2010).

Axenity of decapsulated cysts and *Artemia* culture at the end of each experiment was checked by plating 100 µL of the culture medium on marine agar 2216 in two replicates (Difco, Detroit, USA) and incubation for five days at 28.0 °C. In case of contamination cultures were discarded and the treatment was repeated.

3.2.5. Energy content determination

A subsample of a few g of decapsulated cysts of each H/D treatment was washed carefully with sterile distilled water over a sterile net (50-µm pores); a few hundred mg of these cysts was then oven-dried at 60.0 °C for 24 h. The remaining cysts were transferred to sterile 500-mL hatching bottles containing 400 mL of FASW. The bottles were incubated at 28.0 ± 0.5 °C and constantly exposed to light. After 24 h, the hatched nauplii were harvested and oven-dried at 60.0 °C for 24 h. Energy content of decapsulated cysts and nauplii was analyzed on one replicate sample of approximately 0.5 g dry material per treatment group using a bomb calorimeter (C-7000, Ika, Heitersheim, Germany) at the Particle and Interfacial Technology Group, Faculty of Bioscience Engineering, Ghent University, Belgium. The determination was carried out according to the procedure of AOAC (1995). In order to calculate the individual cyst energy content, firstly the number of cysts per gram dry weight was determined by counting the cysts under the microscope for three replicate samples of 1 mg. Then the energy content of decapsulated cysts per gram dry weight was divided by the average (out of three replicates) of the number of cysts per gram dry weight, resulting in the individual cyst energy content.

3.2.6. Fatty acid analysis

To reduce analytical costs, for each strain only the raw sample and the A222 treatment (which corresponded with the most extreme H/D treatment) were subjected to fatty acid analysis of decapsulated cysts and a population of nauplii harvested after 24 h of incubation (analysis run in one replicate subsample per H/D treatment). Fatty acid composition was determined by gas chromatography according to a modified procedure of Lepage and Roy (1984). This method involves direct acid catalyzed transesterification of dry samples of 10–150 mg without prior extraction of total fat. Ten percent of an internal standard 20:2(n-6) was added before the reaction. Fatty acid methyl esters (FAME) were extracted with hexane. After evaporation of the solvent, the FAME was prepared for injection by redissolving it in iso-octane (2 mg mL^{-1}). Quantitative determination was done by a Chrompack CP9001 gas chromatograph equipped with an auto sampler and a temperature programmable on-column injector. Identification was based on standard reference mixtures (Nu-Chek-Prep, Inc., USA) (Lepage and Roy, 1984). Integration and calculations were done using the software program Maestro (Chrompack).

3.2.7. Vitamin C analysis

Vitamin C analysis was performed (analysis run in one replicate) on the same limited set of samples as used for fatty acid analysis Vitamin C was determined by a paired-ion, reversed phase, high-performance liquid chromatography (HPLC) procedure coupled with electrochemical detection and internal standard quantization based on isoascorbic acid (IAA), and the analysis was carried out according to the procedure of Nelis et al. (1997). The HPLC apparatus consisted of a Varian 8500 pump (Varian Assoc., Palo Alto, CA, USA), an N60 valve injector fitted with a 20-

µL loop (Valco, Houston, TX, USA), and a Coulochem 5100A electrochemical detector (ESA, Inc., Bedford, MA, USA) equipped with a model 5010 or 5011 analytical cell (Nelis et al., 1997).

3.2.8. Statistical analysis

Hatching and survival percentages data were Arcsin transformed to meet normal distribution and homoscedasticity requirements of the residuals (using Levene's test) before further statistical analysis. For each strain and different duration of storage, the data of hatching percentage for 24 h were subjected to one-way ANOVA to detect an effect of the hydration/dehydration treatments. Similarly, for each strain and hydration/dehydration treatment, the data of hatching percentage for 24 h were subjected to one-way ANOVA to detect an effect of the storage period. Additionally for each strain survival data after 12, 24, 36 and 48 h of starvation of metanauplii were each subjected to a one-way ANOVA to detect an effect of the hydration/dehydration treatments. Finally for each strain also the values for number of cysts per gram were subjected to a one-way ANOVA to detect an effect of the hydration/dehydration treatments. For all one-way ANOVA's $P < 0.05$ was considered as significant. A two-factor ANOVA test (SPSS, version 12.0) was used to detect significant interactions between the duration of the hydration period (2 or 4 h) and the number of H/D cycles (1, 2 and 3 cycles) for hatching and survival percentages, and $P < 0.05$ was considered as significant. Tukey test was used to detect significant differences between the experimental sample means, and $P < 0.05$ was considered as significant. Linear regression was used to determine the relationship between parameters in the H/D experiment and $P < 0.05$ was considered as significant. For this analysis, hatching percentages after different period of storage were linearly regressed against the energy content of the cysts exposed to various H/D cycles using a scatterplot in Microsoft Excel. Similarly, survival percentages after different periods of starvation of

metanauplii were linearly regressed against the energy content of nauplii after exposure of cysts to various H/D cycles. Per strain and storage period, pooled standard error of means (PSEM) of hatching percentage was calculated using the formula PSEM = √MSE/n (whereby MSE is mean square of groups, and n is number of observations), pooling the values obtained after different H/D cycles. Similarly for each strain PSEM was calculated for the survival percentages found after a starvation period of 12, 24, 36 and 48 h.

3.3. Results

3.3.1. Hatching characteristics

The hatching percentage of the raw material was 90.1 ± 0.3 % for GSL and 95.1 ± 1.2 % for VC. The hatching curve (Fig. 3.1) shows that T_0 (= incubation time till appearance of first free swimming nauplii) and T_{10} (= incubation time till appearance of 10 % of total hatchable nauplii), which are calculated as a percentage from the maximum hatchability, were the same for GSL and VC, *i.e.* 10 h for T_0 and 10 – 12 h for T_{10}. The cysts showed 90 % of their maximum hatchability (T_{90}) at 18 h for both GSL and VC. The hatching synchrony ($T_S = T_{90} – T_{10}$) was not different between the two strains (= 6 – 8 h for GSL and VC) (Fig. 3.1).

Successive hydration/dehydration cycles increasingly affected the hatching percentage. For GSL cysts of the A1 group, H % was 84.8 % on day zero, 80.6 % after one week and 79.6 % after one month of storage. H % values for the equivalent VC samples were very similar (Table 3.1). For each strain H % of the untreated cysts was significantly higher than that of H/D cysts regardless of the number and duration of H/D cycles and the duration of storage, except for A1/day 0 GSL cysts and A1/1 month VC cysts, where the difference with the untreated cysts was not significant. For GSL cysts, hatchability significantly decreased (Table 3.1) with increasing hydration time and

H/D cycles; this decrease was much more prominent for the samples of the A2 series than for the A1 samples, finally resulting for the A222 group in hatching percentages of 42.7 % on day zero, 41.6 % after one week, and 36.4 % after one month of storage. Interaction between the duration of hydration and the number of H/D cycles was significant, when analyzing the hatching percentage values before storage, and after one week and one month of storage ($P < 0.05$). The results for the VC cysts showed a similar trend (Table 3.1). Storage always resulted in loss of hatching, though the decrease was generally not significant (Table 3.1).

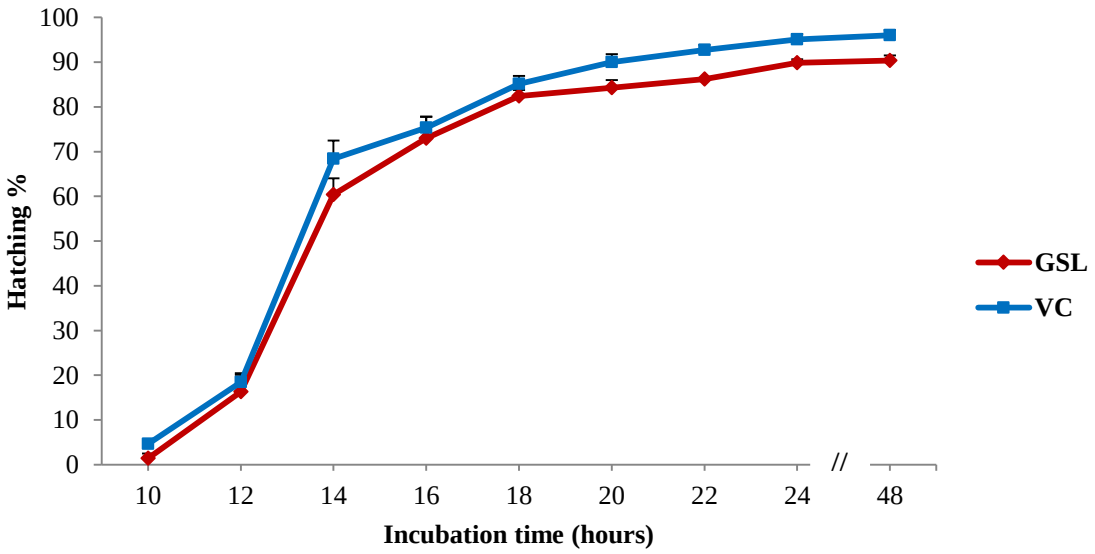

Figure 3.1: Hatching curves of untreated cysts from Great Salt Lake (GSL) and Vinh Chau (VC). Mean values and standard deviation (error bars) of three replicates.

Table 3.1: Hatching percentage (after 24-h hatching) of cysts from GSL and VC strains previously exposed to different duration of the hydration period (2 or 4 h) and different number of H/D cycles (1, 2 and 3 cycles).

Strain	Treatment	Hatching percentage after storage at - 18°C for different durations		
		day 0 (prior to storage)	1 week	1 month
GSL	**Control**	90.1 ± 0.3^{aA}	89.1 ± 1.1^{aA}	87.7 ± 2.2^{aA}
	A1	84.8 ± 0.2^{abA}	80.6 ± 1.8^{bB}	79.6 ± 2.5^{bB}
	A11	79.1 ± 2.6^{bA}	78.9 ± 3.3^{bA}	78.7 ± 0.8^{bA}
	A111	77.3 ± 2.5^{bA}	76.0 ± 1.7^{bA}	75.5 ± 1.4^{bA}
	A2	66.7 ± 2.5^{cA}	64.0 ± 1.2^{cA}	61.9 ± 2.2^{cA}
	A22	55.8 ± 1.3^{dA}	54.1 ± 1.4^{dA}	52.9 ± 2.3^{dA}
	A222	42.7 ± 5.9^{eA}	41.6 ± 3.8^{eA}	36.4 ± 5.7^{eA}
	***Pooled SEM**	± 1.6	± 1.3	± 1.6
	****Interaction**	P = 0.010	P = 0.001	P = 0.000
VC	**Control**	95.1 ± 1.2^{aA}	92.7 ± 0.8^{aAB}	91.2 ± 1.1^{aB}
	A1	88.9 ± 0.2^{bA}	87.6 ± 1.7^{bA}	87.5 ± 1.7^{abA}
	A11	85.0 ± 1.9^{cA}	83.9 ± 1.4^{cA}	83.2 ± 1.0^{bcA}
	A111	83.8 ± 0.8^{cA}	82.3 ± 0.9^{cAB}	80.9 ± 1.1^{cB}
	A2	64.2 ± 1.2^{dA}	61.8 ± 1.7^{dAB}	60.0 ± 1.3^{dB}
	A22	52.8 ± 0.8^{eA}	50.8 ± 1.2^{eA}	50.6 ± 1.1^{eA}
	A222	42.9 ± 0.8^{fA}	40.9 ± 0.1^{fA}	35.7 ± 3.1^{fB}
	***Pooled SEM**	± 0.6	± 0.7	± 0.9
	****Interaction**	P = 0.000	P = 0.000	P = 0.000

For each strain and different duration of storage, small superscripts in each column show significant difference between different hydration/dehydration treatments (one-way ANOVA). For each strain and each hydration/dehydration treatment, capital superscripts in each row show significant differences between different duration of storage (one-way ANOVA). ****** = Interaction between duration of hydration (2 or 4 h) and number of H/D cycles (1, 2 or 3 cycles) (two-way ANOVA). Data are mean value (n = 3) ± standard deviation and ***** = pooled standard error of means (pooled SEM). Significance level was set at $P < 0.05$.

GSL = Great Salt Lake. VC = Vinh Chau. A1 = 2h hydration + 24 h dehydration (1 cycle). A2 = 4 h hydration + 24 h dehydration (1 cycle), A11 = 2 h hydration + 24 h dehydration (2 cycles). A22 = 4 h hydration + 24 h dehydration (2 cycles), A111 = 2 h hydration + 24 h dehydration (3 cycles). A222 = 4 h hydration + 24 h dehydration (3 cycles).

3.3.2. Survival of starved nauplii under axenic conditions

At the first observation (12 h post-hatching) nauplii of the control group (both for GSL and VC) showed the highest survival (78.8 and 86.3 %, respectively) (Table 3.2); in the treatment groups survival was inversely proportional with the number of H/D cycles and especially with the duration of the hydration period (2 h versus 4 h). Interaction between the duration of the hydration period (2 h and 4 h) and the number of H/D cycles was only significant ($P < 0.05$) for the survival at 12 h of the GSL strain. All groups of nauplii showed increasing mortality throughout the 48 h observation period. This resulted in a significantly lower ($P < 0.05$) GSL and VC survival for all A2 treatments as compared to the control for any moment of observation. Also for the A1 treatments the divergence from the control value grew as the number of H/D cycles increased. Overall the discrepancy between the treated groups and the control grew as the nauplii grew older. Overall performance of the VC nauplii (control and treatments) was better than for the GSL sample (Table 3.2).

Table 3.2: Survival (%) over a 48 h period of starved nauplii hatched under axenic condition from GSL and VC cysts previously exposed to different duration of the hydration period (2 or 4 h) and different number of H/D cycles (1, 2 and 3 cycles).

Strain	**GSL**				**VC**			
Treatment	**12 h**	**24 h**	**36 h**	**48 h**	**12 h**	**24 h**	**36 h**	**48 h**
Control	78.8 ± 6.3^{a}	63.8 ± 6.3^{a}	42.5 ± 8.7^{a}	15.0 ± 4.1^{a}	86.3 ± 8.5^{a}	68.8 ± 11.1^{a}	50.0 ± 8.2^{a}	20.0 ± 5.8^{a}
A1	75.0 ± 4.1^{a}	60.0 ± 7.1^{a}	38.8 ± 7.5^{a}	12.5 ± 2.9^{a}	83.8 ± 4.8^{a}	65.0 ± 10.8^{ab}	46.3 ± 6.3^{a}	18.8 ± 4.8^{ab}
A11	72.5 ± 2.9^{a}	57.5 ± 6.5^{a}	35.0 ± 5.8^{a}	10.0 ± 4.1^{ab}	80.0 ± 4.1^{a}	61.3 ± 11.1^{abc}	41.3 ± 6.3^{a}	16.3 ± 4.8^{abc}
A111	70.0 ± 5.8^{a}	53.8 ± 6.3^{ab}	32.5 ± 6.5^{ab}	8.8 ± 2.5^{abc}	77.5 ± 2.9^{ab}	58.8 ± 9.5^{abc}	38.8 ± 4.8^{a}	15.0 ± 4.1^{abc}
A2	55.0 ± 7.1^{b}	41.3 ± 4.8^{bc}	20.0 ± 4.1^{bc}	3.8 ± 2.5^{bcd}	65.0 ± 4.1^{bc}	45.0 ± 4.1^{bcd}	25.0 ± 4.1^{b}	7.5 ± 5.0^{bcd}
A22	40.0 ± 8.2^{c}	28.8 ± 8.5^{cd}	18.8 ± 7.5^{bc}	2.5 ± 2.9^{cd}	51.3 ± 7.5^{cd}	40.0 ± 9.1^{cd}	18.8 ± 6.3^{b}	5.0 ± 7.1^{cd}
A222	30.0 ± 5.8^{c}	22.5 ± 5.0^{c}	12.5 ± 2.9^{c}	1.3 ± 2.5^{c}	41.3 ± 8.5^{d}	30.0 ± 9.1^{d}	13.8 ± 4.8^{b}	2.5 ± 2.9^{d}
***Pooled SEM**	± 3.0	± 3.2	± 3.2	± 1.6	± 3.1	± 4.8	± 3.0	± 2.5
****Interaction**	P = 0.031	P = 0.236	P = 0.826	P = 0.885	P = 0.102	P = 0.724	P = 0.870	P = 0.961

For each strain, different hydration/dehydration treatment and different starvation period (12, 24, 36 and 48 h), small superscripts in each column show significant difference between different treatments (one-way ANOVA). **** =** Interaction between duration of hydration (2 or 4 h) and number of H/D cycles (1, 2 or 3 cycles) (two-way ANOVA). Data are mean (n = 4) ± standard deviation and ***** = pooled standard error of means (pooled SEM). Significance level was set at $P < 0.05$. For abbreviations, see Table 3.1.

3.3.3. Energy content

The number of cysts per gram dry weight and the energy content of hydrated/dehydrated decapsulated cysts and nauplii (joule/g dry weight) were higher for VC than for GSL, but the individual cyst energy content was lower in VC cysts, which are smaller than GSL cysts (Table 3.3). Moreover, in both strains the individual energy content was higher in the control than in the hydrated/dehydrated cysts, with a gradual decrease in energy content as the number of H/D cycles and the duration of the hydration period increased, illustrating that the cysts consume energy while being hydrated. The rate of energy loss during exposure to the various H/D treatments was similar for both strains (Table 3.3). In both strains, cysts lost more energy when being hydrated for 4 h, than when being hydrated twice for 2 h (*e.g.* 8.9 % versus 3.7 % and 9.3 % versus 3.7 % for A2 and A11, respectively, VC and GSL cysts). In both strains, a double or a triple H/D cycle did not result in a two- or threefold decrease of individual energy content.

3.3.4. Nutrient composition

Fatty acid (and vitamin C) levels are presented on a dry weight basis in Table 3.4 for both cysts and nauplii. In Table 3.5 they are presented based on individual cysts and nauplii; for the individual dry weight of the control nauplii, the values reported by Vanhaecke et al. (1983) were used, *i.e.* 2.42 µg for GSL. For VC the value of San Francisco Bay *Artemia* was used, *i.e.* 1.63 µg. A total of 28 fatty acids were recorded (Table 3.5). The saturated fatty acids (SFA) were dominated by 16:0 and 18:0 in the two strains. Among the monounsaturated fatty acids (MUFA), 18:1n-9 was the most abundant in the two strains with control values of 0.070 µg nauplius^{-1} and 0.101 µg cyst^{-1} in the GSL, higher than the 0.037 µg nauplius^{-1} and 0.061 µg cyst^{-1} obtained in the VC (Table 3.5). The treated cyst samples (A222) showed a decrease in this fatty acid of 13 % and 15 % for

GSL and VC strain, respectively, as compared to the control cysts (Table 3.5). Of all HUFAs 18:3n-3 dominated in the GSL A222 and control cyst samples with a range of 0.147 – 0.161 µg cyst^{-1}. In GSL control nauplii this value was 0.124 µg nauplius^{-1} DW. In VC A222 and control cysts 20:5n-3 was the most abundant PUFA with 0.046 – 0.051 µg cyst^{-1} and 0.032 µg nauplius^{-1} DW in the control nauplii.

Though variations were found among the individual fatty acids, in general total (n-3), total (n-6) and total fatty acids were higher in the individual control cysts than in the corresponding nauplii samples, except in case of total (n-3) for GSL. In addition, total fatty acid levels of the samples exposed to H/D cycles were lower than in the control in both cysts and nauplii, but there was no effect of the H/D cycles on the levels, expressed per individual cyst, of some highly unsaturated fatty acids, such as docosahexaenoic acid (DHA, 22:6n-3) in GSL and VC strains. Conversely for other fatty acids, such as EPA (20:5n-3), arachidonic acid (ARA, 20:4n-6) or linolenic acid (18:3n-3), as compared to the control cysts there was a decrease of fatty acid contents per cyst in A222 and a further decrease in the individual nauplius, illustrating that net breakdown of specific HUFAs occurred during the hydration/dehydration process and a further breakdown when the nauplius eventually hatches (Table 3.4 and 3.5).

3.3.5. Vitamin C content

Considerably higher vitamin C values, expressed as ascorbic acid on a dry weight basis, were found in the control nauplii (0.676 and 0.803 mg g^{-1} DW) than in the control cysts (0.289 and 0.367 mg g^{-1} DW), for VC and GSL, respectively (Table 3.4). However, the three H/D cycles with 4 h hydration (A222) decreased the vitamin C content, expressed per individual cyst (Table 3.5) with

60 – 70 % in GSL and VC cysts, respectively and about 10 % in A222 nauplii (mg g^{-1} DW) in both strains as compared to its control (Table 3.4 & 3.5).

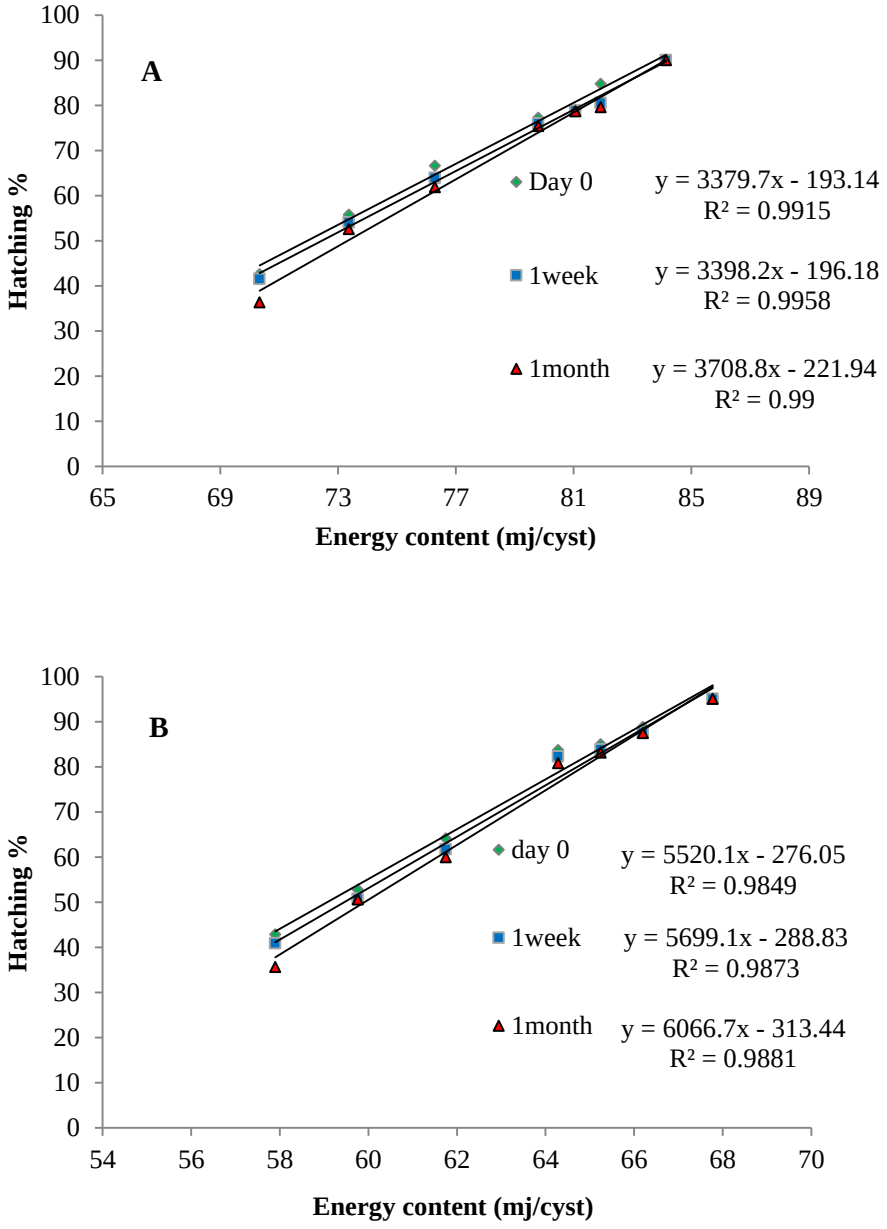

Figure 3.2: (**A**) Linear regression between hatching percentage (on day 0, after 1 week, and 1 month of storage) and energy content (mj $cyst^{-1}$) in **GSL** cysts exposed to various H/D cycles. (**B**) Linear regression between hatching percentage (on day 0, after 1 week, and 1 month of storage) and energy content (mj $cyst^{-1}$) in **VC** cysts exposed to various H/D cycles.

Table 3.3: Energy content of decapsulated GSL and VC *Artemia* cysts previously exposed to different duration of the hydration period (2 or 4 h) and different number of H/D cycles (1, 2 and 3 cycles), and of their corresponding nauplii; percentage loss of individual energy during the H/D cycles as compared to the control, and number of cysts per gram dry weight.

Strain	**GSL**					**VC**				
treatment	Energy content of decapsulated cysts (joule g^{-1} DW)	Energy content of nauplii (joule g^{-1} DW)	Individual energy content (joule $cyst^{-1}$)	Energy loss/cyst (%)	Number of cysts g^{-1} DW	Energy content of decapsulated cysts (joule g^{-1} DW)	Energy content of nauplii (joule g^{-1} DW)	Individual energy content (joule $cyst^{-1}$)	Energy loss/cyst (%)	Number of cysts g^{-1} DW
Control	22.807	22.297	0.0841	0.0	271,070 ± 805^{g}	23.186	22.631	0.0678	0.0	342,100± 900^{g}
A1	22.504	21.956	0.0819	2.6	274,693 ± 606^{f}	22.851	22.330	0.0662	2.3	345,180 ± 815^{f}
A11	22.385	21.750	0.0819	3.7	276,133 ± 1000^{e}	22.651	22.010	0.0653	3.7	347,147 ± 938^{e}
A111	22.196	21.549	0.0798	5.2	278,147 ± 531^{d}	22.508	21.831	0.0643	5.2	350,113 ± 821^{d}
A2	21.669	20.896	0.0763	9.3	284,023 ± 921^{c}	22.050	21.470	0.0618	8.9	357,077 ± 936^{c}
A22	21.215	20.427	0.0733	12.8	289,143 ± 535^{b}	21.651	21.231	0.0598	11.8	362,270 ± 661^{b}
A222	20.707	19.990	0.0703	16.4	294,407 ± 472^{a}	21.331	20.791	0.0579	14.6	368,410 ± 618^{a}

For each strain and number of cysts per gram dry weight, small superscripts in each column show significant difference between different treatments (one-way ANOVA). Energy values correspond with one measurement of energy content per sample; data for number of cysts are mean value ± standard deviation (n = 3). Significance level was set at $P < 0.05$. DW = dry weight. For abbreviations see Table 3.1

Table 3.4: Fatty acid methyl esters (FAME) composition (mg g^{-1} DW) and vitamin C contents (mg g^{-1} DW) in control and A222 decapsulated cysts and in the corresponding nauplii of GSL and VC *Artemia* strain. Values correspond with one single analysis. For abbreviations, see Table 3.1.

Strain	**GSL**				**VC**			
	Cysts		**Nauplii**		**Cysts**		**Nauplii**	
FAME	**Control**	**A222**	**Control**	**A222**	**Control**	**A222**	**Control**	**A222**
14:0	1.3	1.1	0.9	0.9	3.3	3.0	3.0	2.9
14:1n-5	1.7	1.6	1.7	1.6	1.1	1.0	1.1	1.1
15:0	0.3	0.3	0.2	0.2	6.1	5.7	5.6	5.6
15:1n-5	0.7	0.3	0.8	0.3	0.6	0.6	0.6	0.6
16:0	17.0	16.5	16.2	15.7	19.4	18.0	18.9	18.3
16:1n-7	3.8	3.7	3.2	3.0	17.0	15.8	17.0	16.9
17:0	1.1	1.0	1.1	1.0	6.7	4.8	7.0	5.4
17:1n-7	1.4	1.3	1.3	1.2	2.1	1.7	2.0	1.9
18:0	7.1	6.7	5.6	7.8	4.8	4.4	5.4	5.1
18:1n-9	27.5	25.8	27.3	26.2	20.9	19.2	22.8	20.7
18:1n-7	8.5	8.2	8.8	8.7	14.5	13.3	15.1	15.6
18:2n-6^{t}	0.4	0.4	0.4	0.4	0.3	0.1	0.4	0.4
18:2n-6^{s}	9.2	9.0	10.0	9.9	4.4	4.1	4.8	4.7
19:0	0.1	0.1	0.1	0.1	0.0	0.0	0.0	1.5
18:3n-6	1.3	1.3	1.4	1.4	1.3	1.3	1.3	1.3
19:1n-9	0.5	0.4	0.5	0.5	1.3	1.2	1.5	1.5
18:3n-3	43.6	43.4	51.4	51.8	3.8	3.6	4.4	4.3
18:4n-3	9.8	9.8	11.5	11.7	1.6	1.4	1.5	1.4
20:0	0.1	0.1	0.2	0.2	0.4	0.1	0.3	0.1
20:1n-9	0.7	0.6	1.0	0.9	1.4	1.2	0.8	1.2
20:1n-7	0.1	0.1	0.2	0.1	0.2	0.2	1.9	0.2
20:3n-6	0.2	0.1	0.2	0.2	0.5	0.4	0.5	0.5
20:4n-6	0.4	0.4	0.3	0.3	6.0	5.6	6.7	6.5
20:3n-3	1.5	1.4	2.2	2.2	0.1	0.1	0.2	0.1
20:4n-3	1.6	1.6	2.2	2.2	0.7	0.6	0.6	0.9
22:0	0.3	0.3	0.6	0.5	0.3	0.1	0.4	0.4
20:5n-3	1.6	1.6	1.2	1.2	17.6	17.0	19.9	19.6
22:6n-3	0.0	0.0	0.1	0.0	0.2	0.0	0.2	0.0
Total (n-3)	4.9	4.8	6.1	5.8	19.0	18.0	21.6	21.0
Total (n-6)	11.5	11.2	12.3	12.2	12.7	11.6	13.9	13.5
Total (SFA)	27.3	26.1	24.9	26.4	41.0	36.1	40.6	39.3
Total MUFA	44.9	42.0	44.8	42.5	59.1	54.2	62.8	59.7
Total PUFA	69.6	69.0	80.9	81.3	36.5	34.2	40.5	39.7
Total FAME	153.3	149.3	165.8	162.9	171.9	166.5	182.1	173.7
Vitamin C	0.367	0.112	0.803	0.724	0.289	0.111	0.676	0.611

Table 3.5: Fatty acid methyl esters (FAME) composition (µg cyst^{-1} DW) and vitamin C contents (µg cyst^{-1} DW) in control and A222 decapsulated cysts and in the corresponding control nauplii (µg nauplius^{-1} DW) of GSL and VC *Artemia* strain. Values correspond with one single analysis. For abbreviations, see Table 3.1.

Strain	**GSL**			**VC**		
	Cysts		**Nauplii**	**Cysts**		**Nauplii**
FAME	**Control**	**A222**	**Control**	**Control**	**A222**	**Control**
14:0	0.005	0.004	0.002	0.010	0.008	0.005
14:1n-5	0.006	0.005	0.004	0.003	0.003	0.002
15:0	0.001	0.001	0.001	0.018	0.015	0.009
15:1n-5	0.003	0.001	0.002	0.002	0.002	0.001
16:0	0.060	0.056	0.400	0.057	0.049	0.031
16:1n-7	0.010	0.013	0.008	0.050	0.043	0.028
17:0	0.004	0.003	0.003	0.020	0.013	0.015
17:1n-7	0.005	0.004	0.003	0.006	0.005	0.003
18:0	0.030	0.023	0.013	0.014	0.012	0.009
18:1n-9	0.101	0.088	0.070	0.061	0.052	0.037
18:1n-7	0.031	0.023	0.021	0.042	0.036	0.025
18:2n-6^{t}	0.001	0.001	0.001	0.001	0.000	0.001
18:2n-6^{s}	0.034	0.031	0.024	0.013	0.011	0.008
19:0	0.000	0.000	0.000	0.000	0.000	0.000
18:3n-6	0.005	0.004	0.003	0.004	0.004	0.002
19:1n-9	0.002	0.001	0.001	0.004	0.003	0.003
18:3n-3	0.161	0.147	0.124	0.011	0.010	0.007
18:4n-3	0.036	0.033	0.028	0.005	0.004	0.003
20:0	0.000	0.000	0.001	0.001	0.000	0.001
20:1n-9	0.003	0.002	0.002	0.004	0.003	0.001
20:1n-7	0.000	0.000	0.001	0.001	0.001	0.003
20:3n-6	0.000	0.000	0.001	0.002	0.001	0.001
20:4n-6	0.002	0.001	0.001	0.018	0.015	0.011
20:3n-3	0.006	0.005	0.005	0.000	0.000	0.000
20:4n-3	0.006	0.005	0.005	0.002	0.002	0.001
22:0	0.001	0.001	0.002	0.001	0.000	0.001
20:5n-3	0.006	0.005	0.003	0.051	0.046	0.032
22:6n-3	0.000	0.000	0.000	0.001	0.000	0.000
Total (n-3)	0.018	0.016	0.020	0.056	0.049	0.035
Total (n-6)	0.042	0.038	0.030	0.040	0.031	0.023
Total (SFA)	0.101	0.089	0.060	0.120	0.098	0.070
Total MUFA	0.166	0.143	0.108	0.173	0.147	0.102
Total PUFA	0.257	0.234	0.200	0.107	0.093	0.070
Total FAME	0.566	0.507	0.401	0.502	0.452	0.300
Vitamin C	0.001	0.0004	0.001	0.001	0.0003	0.001

3.3.6. Correlations between quality parameters

Linear regression analysis between hatching percentage (on day 0 and after one week and one month of storage) on the one hand, and individual energy content of cysts on the other, indicated a very strong positive correlation in both GSL and VC strains ($R^2 > 0.99$ for GSL and $R^2 > 0.98$

for VC (Fig. 3.2 A and B), A similar positive correlation was found between survival of the starved nauplii and the energy content (expressed in joule g^{-1} dry weight) of nauplii ($R^2 > 0.95$ for GSL and $R^2 > 0.92$ for VC (Fig. 3.3 A and B). There were no significant differences ($P > 0.05$) between the slopes and between the intercepts of the regression lines in Figures 3.2 A and B, whereas in both Figure 3.3 A and B all slopes and intercepts of the four regression lines were significantly different ($P < 0.05$) from each other.

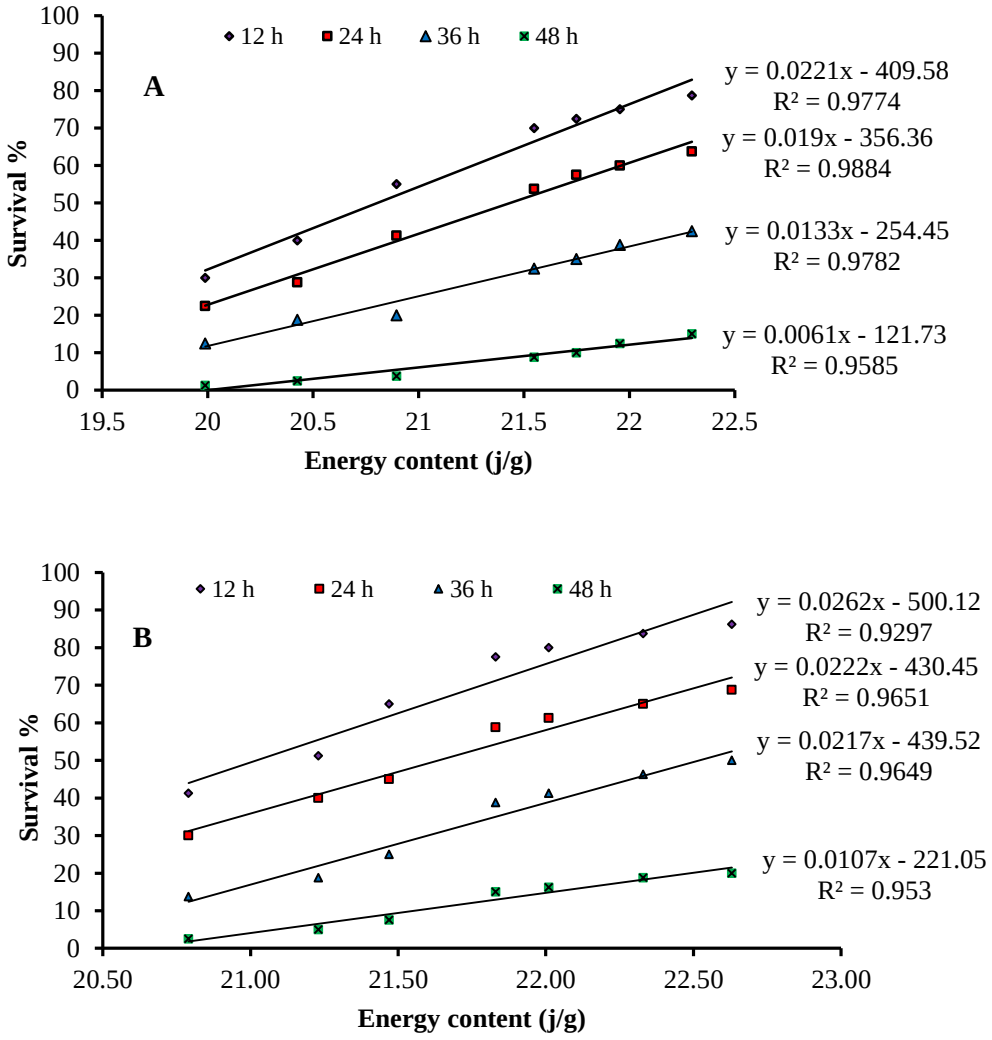

Figure 3.3: (**A**) Linear regression between survival % (after 12, 24, 36, and 48 h) and energy content (j g^{-1} DW) of **GSL** nauplii after exposure of the cysts to various H/D cycles. (**B**) Linear regression between survival % (after 12, 24, 36, and 48 h) and energy content (j g^{-1} DW) of **VC** nauplii after exposure of the cysts to various H/D cycles.

3.4. Discussion

The ability of *Artemia* to form cysts accounts in part for its convenience as a larval food source (Léger et al. 1986). *Artemia* cysts have a remarkable shelf life; the ease and simplicity of hatching make brine shrimp one of the most convenient, least labor-intensive live foods available for aquaculture.

This study aimed to gauge to what extent cyclic H/D exposure of cyst samples from two commercially important *A. franciscana* strains (Great Salt Lake, USA, and the San Francisco Bay-type (SFB) Vinh Chau, Vietnam) resulted in quality loss as assessed by aquaculture-relevant parameters such as hatching, longevity and nutritional quality of nauplii.

The untreated cyst samples used in our study were in quiescence, as illustrated by the high hatching percentage (> 90 %). The hatching quality of the cysts was negatively affected when cysts were stored after exposure to a succession of H/D cycles, and this effect aggravated as the number of cycles increased from one to three, and as the hydration period, preceding dehydration, was lengthened from 2 to 4 h, finally resulting in a loss of hatching in the range 50 – 60 % of the control value. The loss as a result of multiple H/D cycles was more marked when a hydration period of 4 h was used, as compared with hydration during 2 h. The difference in water content of the VC and GSL control sample (34.8 % versus 4.5 %, respectively) did not affect these results: after incubation in seawater, cysts absorb water at a fast rate, reaching a maximal water content of 140 % of their dry weight after 1.0 – 1.5 h at 28.0 °C, as described by (Lavens and Sorgeloos, 1987).

A similar effect was found for the axenically hatched nauplii with survival negatively affected by increasing H/D cycles, longer hydration incubation, and prolonged starvation over a 48 h observation period, as energy reserves were gradually depleted (Benijts et al. 1976). Analogously

the individual energy content, being maximal in the control cysts, gradually declined as the number of H/D cycles and the duration of the hydration period increased, corresponding with energy consumption during the repeated hydration process (Morris, 1971; Vanhaecke and Sorgeloos, 1982; Vanhaecke et al. 1983). Clegg (1964) reported that carbohydrates are the main stored energy reserves and it is the disaccharide trehalose that forms the bulk of this reserve prior to hatching. Previous studies have also suggested that cysts exposed for too long a period to water levels exceeding 65 % will initiate the utilization of trehalose, through conversion into glycogen and glycerol as a source of energy for embryonic development which eventually results in energy depletion (Clegg, 1976; Lavens and Sorgeloos, 1987). In both strains hydration for 4 h resulted to more than three times (3.5 – 3.8 times) the energy loss resulting from hydration for 2 h, illustrating that as hydration continues, metabolism intensifies and energy consumption proceeds at a faster rate (Lavens and Sorgeloos, 1987). On the other hand, doubling or tripling the H/D cycle did not result in an accordingly double or triple energy loss. This suggests that after the first cycle, a number of metabolic mechanisms have been initiated, which are not repeated during the following cycles. The similarity in energy consumption during the successive H/D cycles, observed in both strains, suggests that the underlying mechanisms are common within the species *A. franciscana*, and possibly within the genus *Artemia*. Nevertheless, as energy content in the cysts dropped, cyst hatching and naupliar survival decreased faster in VC than in GSL, as reflected in the slope of the regression curves (Figs 3.2 A, 3.3 B), indicating that VC cysts are more sensitive to improper storage conditions than GSL, which may be linked to their smaller size (generally with a diameter of approximately 225 µm for VC, as compared to about 250 µm for GSL cysts (Vanhaecke and Sorgeloos, 1980; Dhont and Sorgeloos, 2002).

Comparative literature information on the fatty acid contents of cysts and the nauplii emerging from them is scarce (Garcia-Ortega et al. 1998; Dhont and Sorgeloos, 2002) and no information is available on the fatty acid metabolism involved in the hatching process. Our FAME results of control decapsulated cysts and nauplii of the VC and GSL strains, and the differences in dominating fatty acids between both strains, are similar to the literature data published for those strains (Evjemo et al. 1997; Garcia-Ortega et al. 1998; Dhont and Sorgeloos 2002; Ando et al. 2002). The effect of hydration and subsequent dehydration on the fatty acid contents of cysts has not been the subject of systematic studies in the past. In our study, total FAME levels, when expressed on a dry weight basis, were slightly lower in A222 cysts than in the control cysts (in the order 2.6 – 3.1 % for both strains), and the same was the case when comparing A222 nauplii and controls (decrease in the range 1.7 – 4.6 %). However, when expressed as FAME contents of individual cysts, the decrease was about 10 % for both strains. In individual control nauplii, the decrease was 30 – 40 % compared to the individual control cysts. This suggests that fatty acids may be used as energy resource during the H/D process of cysts and also further on in the process towards emergence of nauplii. García-Ortega et al. (1998) report that, small changes in fatty acids composition were found during different developmental stages of cysts and early stages of nauplii.

In general, fatty acid analysis showed that the two populations of *Artemia* from Vinh Chau and from Great Salt Lake are essentially different in fatty acid composition, especially in terms of EPA (20:5n-3) and linolenic acid (18:3n-3) contents. Variations in cyst fatty acid profile are generally linked to the characteristics of the phytoplankton population as food source for the maternal population (Navarro et al. 1992ab; Zhucova et al. 1998; Thinh et al. 1999, Torrentera and Dodson, 2004), though also other environmental parameters, such as ambient temperature, and genetic factors may have an effect (Ruiz et al. 2007, 2008; Nguyen Thi Hong Van, unpublished results).

In order to meet the nutritional requirements of especially marine fish and shellfish larvae, enrichment of *Artemia* metanauplii is a standard procedure in many hatcheries when using the HUFA-deficient GSL strain. HUFA levels post-enrichment thus overwhelm the levels of the freshly hatched nauplius, or of the metanauplius having gone through some period of starvation. Enrichment is not applied for the VC strain, however, which contains relatively high levels of highly unsaturated fatty acids. Moreover, strict hatching procedures (*e.g.* harvesting of nauplii after a hatching incubation period of 24 h) generally reduce the risk of starved (meta) nauplii being fed to (shell) fish larvae.

Vitamin C levels were conform to the conversion of vitamin C from ascorbic sulphate into ascorbic acid during completion of the embryonic development into the nauplius stage, which explains for the considerably higher values found in nauplii than in cysts (Dabrowski, 1991; Golub and Finamore, 1972; Nelis et al. 1994). Our values in cysts were also comparable to the range (162 – 428 µg g^{-1} DW) reported by Dabrowski (1991) for cysts. The variation in vitamin C contents found between cysts of different geographical origin (in the range 296 – 517 µg g^{-1} DW expressed as ascorbic acid), its conversion into free ascorbid acid during the hatching process, and the role of ascorbic sulphate as storage form have been studied in detail (Mead and Finnamore 1969; Merchie et al. 1995a). The nutritional quality of the A222 cysts and nauplii was lower than in the control, as shown by reduced fatty acid and vitamin C levels. In the case of vitamin C, this loss, expressed per individual cyst, amounted in both strains up to 60 – 70 % for A222 cysts as compared to the control.

Knowledge of hatching characteristics in *Artemia* samples is important due to the reported variability among batches and strains. The nutritional quality in *Artemia* varies considerably as well, in relationship with its geographical origin (Léger et al. 1986). (Vanhaecke and Sorgeloos,

1982) reported that the poor hatchability of commercial batches of *Artemia* cysts can be linked to improper processing of the cysts after collection in nature, and that long-term storage of such material may result in a further substantial decrease in hatching success. In natural conditions cysts may be exposed to H/D cycles, as they are floating driven by wind and currents, and may temporally or definitively accumulate on the shore where they undergo the fluctuations of atmospheric conditions. Harvesting of good quality cyst product requires collection of recently produced cyst batches from the open water shortly followed by adequate processing, but these conditions are seldom fulfilled, especially when harvesting natural production in inland lakes, when site accessibility is limited, timing of harvesting is irregular, transport and storage is an issue, and/or overall expertise is lacking. In *Artemia* pond production, such as in the Mekong Delta, Vietnam, frequent harvesting (up to 2 – 3 times a day) followed by adequate processing is currently done, which contributes to the good quality of the resulting cyst product (Anh et al. 2009).

Our results confirm that cyst metabolism, as initiated after hydration, can be interrupted and that cysts can be converted from a hydrated, metabolically active mass of cells into a dehydrated, ametabolic state. They also show that repeated H/D cycles not only result in a decreased hatching output, but also in an inferior quality of those nauplii hatching, as quantified in our study by naupliar longevity, energy, FAME and vitamin C content. These observations are of fundamental importance in understanding the cysts quality and they have significant potential for application in aquaculture.

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Chapter 4

Hydration-dehydration cycles imposed on *Artemia* cysts influence the tolerance limit of nauplii against abiotic and biotic stressors

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Abstract

The brine shrimp *Artemia franciscana* is an important live feed for fish and shellfish larviculture. Cysts of *Artemia* are naturally found in a wide variety of harsh environments worldwide where they are exposed to different changing environmental conditions. *Artemia* cysts are also exposed to different hydration/dehydration (H/D) conditions during the post-harvest processing period in the *Artemia* processing industry. In this study, cysts of two strains of *A. franciscana* originating from two different geographical locations: one a natural population from Great Salt Lake (GSL), Utah, USA, and the other cultured in the Vinh Chau (VC) salt ponds, Vietnam, where they experience different environmental factors, were exposed to successive hydration/dehydration (H/D) cycles with the aim of determining the effects of these conditions on the stress (*i.e.* induction of Hsp70) and functional (*i.e.* resistance towards abiotic and pathogenic biotic factors) responses of the emerged nauplii. Our results showed that a short period of H/D of the cysts did not appear to have a deleterious effect on the emerged nauplii of both strains, as was evidenced by absence of significant difference in the survival of nauplii emerged from the control and treated cysts, upon challenge with a thermal stressor or with pathogenic *Vibrio campbellii*. A limited exposure to H/D treatment even leads to the induction of enhanced thermotolerance in GSL nauplii. In essence, these observations add some insights to our current understanding of stress responses in *Artemia* under the described experimental conditions. In addition, the impact of H/D cycles associated with stress response should be taken into consideration when *Artemia* is considered as a model organism for future research and applications.

4.1. Introduction

Stress is a state where organismal homeostasis is either threatened or interrupted by intrinsic and/or extrinsic stimuli or stressors (Chrousos and Gold 1992; Mercier et al., 2006). Aquatic organisms are often exposed to a multitude of abiotic and biotic stressors, to which they respond by inducing a cascade of molecular and physiological pathways (Song et al., 2006; Tort 2011). The most common features of the molecular stress response are the induction of heat shock proteins (Hsps). Hsps, mainly the 70 kilodalton Hsp (Hsp70), are evolutionarily conserved protein molecules that are present in, and considered essential to the survival of, all prokaryotic and eukaryotic cells (Lindquist and Craig 1988; Lindquist 1992). The expression of Hsp70 is either constitutive or inducible under different conditions. The constitutive form has a crucial function as molecular chaperone and is involved in the folding of nascent polypeptides, assembly/disassembly of multi-subunit oligomers, translocation of proteins across intracellular membranes, process of endocytosis, regulation of apoptosis and cytoskeletal organization (Ohtsuka et al., 2007). The inducible Hsp70 works to repair partially denatured proteins, to facilitate the degradation of irreversibly denatured proteins and to inhibit protein aggregation, thus protecting cells from harmful environmental stresses (Parsell and Lindquist 1993). In experimental animals, induction of Hsp70 in response to exposure to abiotic and/or pathogenic biotic stressing agents has been shown to play an important role in conferring resistance to the animals against these stressors (Sung et al., 2007, 2011a; Kiss et al., 2011; Baruah et al., 2013, 2014). For instance, in aquaculture animal models, induction of Hsp70 was associated with the protection of the animals against thermal challenge, viral attack, ammonia toxicity, or transportation stress (for details, see review Roberts et al., 2010; Sung et al., 2011b).

The brine shrimp *Artemia franciscana* plays an important role in the diet of fish and shellfish larvae produced in industrial hatcheries (Sorgeloos et al., 1986, 2001). *Artemia* has also been considered as a useful organism for stress response studies (MacRae 2003). This is because they are naturally found in a variety of harsh environments worldwide (Triantaphyllidis et al., 1998) where they are exposed to a variety of changing environmental factors (MacRae 2003; Clegg 2007). For adaptation of its life strategy to these conditions, *Artemia* possesses two reproductive pathways. Under favorable conditions, the female releases free-swimming nauplii (ovoviviparity). Under unfavorable environmental conditions, however, the embryological development is interrupted at the gastrula stage; the embryo is surrounded by a chitinous shell and released in the environment as diapausing eggs or cysts (oviparity) (Van Stappen 1996).

Because of the changing environmental conditions in the natural habitat and eventually also during the period of post-harvest processing in the *Artemia* processing industry, the *Artemia* cysts may pass through several cycles of hydration/dehydration (H/D). This may have severe impact on the quality of cysts in terms of hatching percentage, hatching efficiency and performance of the hatched nauplii (Vanhaecke and Sorgeloos 1982; Lavens and Sorgeloos 1987). To a certain extent, some level of hydration/dehydration exposure is an almost unavoidable element in the history of any commercial cyst sample from pre-harvesting until marketing. In our previous study in which cysts went through several hydration/dehydration cycles, the latter factor was shown to have a marked negative impact on the energy content of the embryo, resulting in the hatching of inferior quality nauplii (*i.e.* decreased cyst hatching, reduced starved naupliar longevity, loss in vitamin C and fatty acid content) (Chapter 3). In the present study, we aimed at investigating the effects of hydration/dehydration cycles of *Artemia* cysts on the stress responses of cysts and emerged nauplii. As marker to measure stress response, we focused on Hsp70 production level in the cysts and

emerged nauplii, and also on survival performance of the emerged nauplii upon challenge by abiotic or pathogenic biotic stressor. To conduct this experiment, we used the gnotobiotic *Artemia* rearing test system, which had been proven useful in various stress response studies, especially in establishing cause-effect relationship of stress-inducing agents (Marques et al., 2006ab; Sung et al., 2007; Baruah et al., 2014). Such system permits eliminating the interference of multiple factors (such as changing microbial communities, rearing conditions) during the experimental period and eventually allows determining the biological responses of the test organism towards the specific testing agent (Marques et al., 2006a).

4.2. Materials and methods

4.2.1. Bacteria strain and culture conditions

The pathogenic *Vibrio campbellii* strain LMG21363 was used for *Artemia* challenge assay (Baruah et al., 2015). This strain has previously been shown to cause massive mortality in *Artemia* cultures (Marques et al., 2006a; Haldar et al., 2011) and it has frequently been used for this type of challenge tests due to its high virulence (Defoirdt et al., 2006a,b; 2007). The bacteria, preserved at - 80 °C in 20 % sterile glycerol, were initially grown at 28 °C for 24 h on Marine Agar (Difco Laboratories, Detroit, MI, USA) and then to the log phase in Marine Broth 2216 (Difco Laboratories, Detroit, MI, USA) by incubation at 28 °C with continuous shaking prior to use. Bacterial cell numbers were determined spectrophotometrically at 550 nm according to the McFarland standard (BioMerieux, Marcy L'Etoile, France), assuming that an OD of 1.000 corresponds to 1.2×10^9 cells mL^{-1} and after cultivation, the pathogen was immediately used.

4.2.2. Artemia cyst samples

Two strains of *A. franciscana* (Kellogg 1906), harvested in 2007, were used in this experiment: (1) commercial dry cysts originating from Great Salt Lake (GSL), Utah, USA (INVE Aquaculture Belgium, Type EG (batch number: 21425); and (2) cysts from Vinh Chau (VC) salt ponds, Vietnam (ARC code 1718), supplied by Can Tho University, Vietnam. These two strains originate from two different geographical locations, where they experience different environmental factors, which may be potentially stressful (*e.g.* temperature stress). In order to maintain maximal viability, both samples had been stored at + 4 °C since their arrival at the Laboratory of Aquaculture & *Artemia* Reference Center.

4.2.3. Hydration/dehydration cycles

For the tests, described in this Chapter, the same cyst samples were used, that had been exposed to the six H/D treatments (named A1, A11, A111 and A2, A22, A222), as described in Chapter 3. After the H/D treatments the cyst samples had been stored in + 4 °C in NaCl-saturated brine (280 – 300 g L^{-1}) for 4 weeks until use for the tests described below.

4.2.4. Axenic hatching of Artemia

Axenic *Artemia* cysts and nauplii were obtained following decapsulation and hatching as described previously (Baruah et al., 2011; Haldar et al., 2011). Briefly, 1 g of *Artemia* cysts of each H/D treatment and of the control were hydrated in 89 mL of distilled water for 1 h. Sterile nauplii were obtained via decapsulation of cysts using 3.3 mL NaOH (32 %) and 50 mL NaOCl (50 %). During the reaction, 0.22 µm filtered aeration was provided. All manipulations were carried out under a laminar flow hood and all tools were autoclaved at 121 °C for 20 min. The decapsulation was

stopped after about 2 min by adding 50 mL of $Na_2S_2O_35H_2O$ (10 g L^{-1}). The aeration was then terminated and the decapsulated cysts were washed with filtered (0.2 µm) and autoclaved artificial seawater containing 35 g L^{-1} of Instant Ocean synthetic sea salt (Aquarium Systems, Sarrebourg, France). The cysts were re-suspended in 500-mL glass bottles containing 400-mL of filtered and autoclaved artificial seawater and incubated for 24 h at 28 °C with constant illumination (approximately 27 µE m^{-2} s^{-1}). All these manipulations were performed under a laminar flow hood in order to maintain sterility of the cysts and nauplii. After 24 h incubation, nauplii were harvested and immediately used in the thermotolerance and challenge assay.

4.2.5. Methods used to verify axenicity of Artemia

After hatching, the axenicity of the *Artemia* nauplii was verified by spread plating 100 µL of the hatching water on Marine Agar (Difco, Detroit, USA) followed by incubating at 28 °C for 5 days (Baruah et al., 2012). Experiments started with non-sterile nauplii were discarded.

4.2.6. Protein extraction and western blot detection of Hsp70

Samples containing 0.1 g of decapsulated H/D cysts or live nauplii emerged from the decapsulated H/D cysts were homogenized in cold buffer K (150 mM sorbitol, 70 mM potassium gluconate, 5 mM $MgCl_2$, 5 mM NaH_2PO_4, 40 mM HEPES, pH 7.4) (Clegg et al., 2000) and supplemented with protease inhibitor cocktail (Sigma-Aldrich, Inc. USA) as recommended by the manufacturer. Subsequent to centrifugation at 2200 x *g* for 1 min at 4 °C, supernatant protein concentrations were determined by the Bradford method (Bradford 1976) using bovine serum albumin as standard. Supernatant samples were then combined with loading buffer, vortexed, heated at 95 °C for 5 min and electrophoresed in 10 % SDS-PAGE gels, with each lane receiving equivalent amounts of

protein (25 µg). Gels were stained with Coomassie Biosafe (BioRad, Belgium) and then transferred to polyvinylidene fluoride membranes (BioRad, Belgium) for antibody probing as described previously (Sung et al., 2007). Membranes were incubated with blocking buffer [50 mL of 1x phosphate buffered saline containing 0.2 % (volume/volume) Tween-20 and 5 % (weight/volume) bovine serum albumin] for 60 min at room temperature and then with mouse monoclonal anti-Hsp70 antibody, clone 3A3 (Affinity BioReagents Inc., Golden, CO), which recognizes both constitutive and inducible Hsp70 (Baruah et al., 2010), at the recommended dilution of 1:5000. Horseradish peroxidase conjugated donkey anti-mouse IgG was used as secondary antibody at the recommended dilution of 1:2500 (Affinity BioReagents Inc., Golden, and CO). The membranes were then treated with enhanced chemiluminescence reagent (BioRad, Belgium) and the signals were detected by a ChemiDoc MP Imaging System (BioRad, Belgium). The signal intensity was quantified by densitometry with Biorad Image Lab™ Software version 4.1., and for the treated *Artemia* cysts and nauplii expressed relative to the control group (Norouzitallab et al., 2014).

4.2.7. Thermotolerance assay

For each H/D treatment and for the control of both strains, 30 *Artemia* nauplii were transferred to new sterile 40-mL glass tubes containing 30 mL of filtered and autoclaved artificial seawater. The animals were subjected to a lethal heat shock by immersing the *Artemia* rearing tubes for 20 min in a water bath preheated to 41 °C (Δt = 5 °C min^{-1}) as described previously (Sung et al., 2007; 2008). Thermal-shocked *Artemia* nauplii were slowly brought back to a water temperature of 28 °C at a Δt rate of 0.5 °C min^{-1}. Thermotolerance was determined by counting the live nauplii 36 h

after thermal challenge. The animals were not fed during this process. Each treatment was carried out in quintuplicate.

4.2.8. Artemia challenge assay

Groups of 30 *Artemia* nauplii hatched from treated and control *Artemia* cysts were transferred to new sterile 40-mL glass tubes containing 30 mL of filtered and autoclaved artificial seawater. The nauplii were then challenged with *Vibrio campbellii* LMG21363 at a concentration of 10^7 cells mL^{-1} as described previously (Sung et al., 2008). The survival of *Artemia* was scored after 36 h. The animals were not fed during this process. Each treatment was carried out in quintuplicate. Bacteria-free status was verified as described above. All procedures were aseptically performed under a laminar flow hood.

4.2.9. Statistical analysis

Survival data were arcsin transformed to satisfy normality and homocedasticity requirements as necessary. To determine the overall effect of each single treatment, data were analyzed by one-way ANOVA using the statistical software Statistical Package for the Social Sciences version 16.0. Tukey test was used to determine significant differences between the means. P value < 0.05 was considered significant.

4.3. Results

4.3.1. Induction of Hsp70 in the cysts and nauplii

To determine whether H/D treatment of the cysts has an inductive effect on Hsps, we analyzed Hsp70 production in the control and treated cysts and also in the emerged nauplii by western blot

assay as described in the methodology. As shown in Fig. 4.1, there was a constitutive production of Hsp70 in the cysts and nauplii of all the groups as indicated by the presence of a band in the control cysts and nauplii of both strains. Using the Hsp70 quantification methodology according to Norouzitallab et al. (2014), there was a marked increase in the GSL cysts in the level of Hsp70 production in all the groups due to H/D treatment compared to the control cysts, with maximum induction recorded in the A22 group (Fig. 4.1 A and 4.1 B, cysts). However, the emerged GSL nauplii from the treated cysts had a lower level of Hsp70 than the control nauplii and the Hsp70 level decreased with increase in the hydration cycle and period (Fig. 4.1 A and 4.1 B, nauplii). In the VC strain, the Hsp70 level in the cysts often decreased with increase in the hydration period and cycles of the cysts. Among the treatments, maximum and minimum Hsp70 levels were observed in A1 and A222, respectively (Fig. 4.1 C and 4.1 D, cysts). The nauplii developed from the treated cysts had lower levels of Hsp70 than the control nauplii (Fig. 4.1 D, nauplii). In both strains, the level of Hsp70 in the cysts was markedly higher than in the emerged nauplii (except for the A2 series in VC).

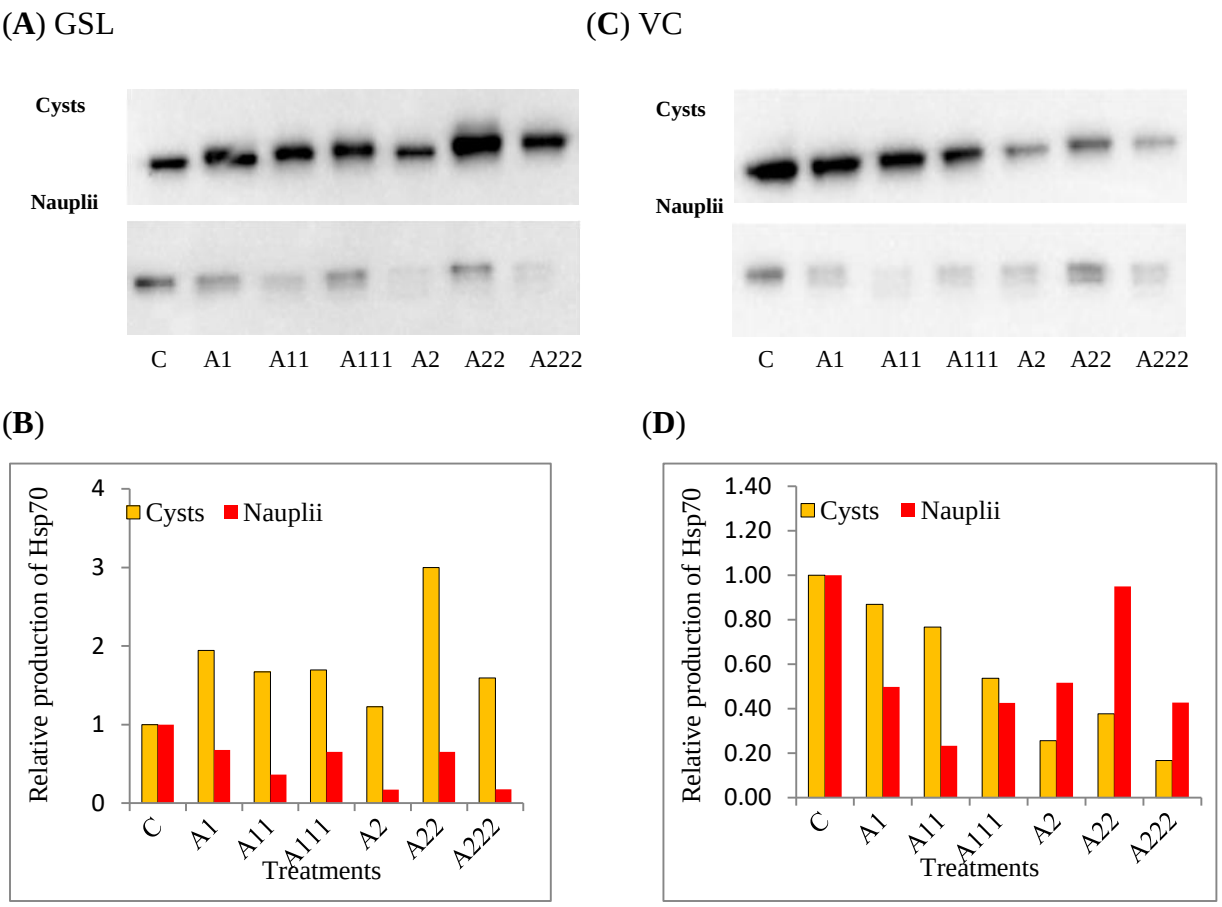

Figure 4.1: Effect of successive hydration/dehydrations cycles on the induction of Hsp70 in the cysts and in emerged nauplii of GSL and VC *Artemia* strains. For the treatment groups, see Table 4.1 for explanation. A, C: Extracted protein resolved in SDS–PAGE gel, transferred to polyvinylidene fluoride membrane and probed with antibody to *Artemia* Hsp70. B, D: Quantitative analysis of Hsp70 in the treated *Artemia* cysts and nauplii, expressed relative to the control (C) group, using Chemi Doc MP Imaging System (Norouzitallab et al., 2010)

4.3.2. Thermotolerance of nauplii hatched from cysts exposed to successive hydration/dehydration treatments

The survival of the A1 and A11 GSL nauplii was significantly higher than that of the control (Table 4.1 A, $P < 0.05$). Increasing the number of H/D cycles to 3 (A111) or prolonging the hydration period from 2 h to 4 h (A2, A22, and A222) significantly reduced the survival of the thermally challenged nauplii, with lowest survival being observed in A222. In case of the VC strain, the survival of the control and of the A1 and A11 *Artemia* nauplii did not differ significantly among

each other, but these values were significantly higher than in all other treatments (Table 4.1 A). As in GSL, there was a decreasing trend A1 > A11 > A111 and A2 > A22 > A222. In each treatment and in the controls, the VC strain was more resistant to thermal stress than GSL.

Table 4.1: Survival (%) of *Artemia* nauplii after 36 h of challenge with (**A**) thermal shock or (**B**) *V. campbellii*.

Treatments	Survival (%)			
	A. Thermotolerance assay		**B. *Vibrio* challenge assay**	
	GSL	**VC**	**GSL**	**VC**
Control	22.6±1.5^{b}(100.0)	41.3±3.8^{a}(100.0)	8.0±1.8^{a}(100.0)	32.7±2.8^{a}(100.0)
A1	34.0±2.8^{a}(150.4)	38.0±3.8^{a}(92.0)	7.3±2.8^{a}(91.7)	29.3±2.8^{a}(89.8)
A11	30.0±3.3^{a}(132.7)	35.3±3.8^{a}(85.5)	6.7±2.4^{ab}(83.3)	27.3±4.3^{a}(83.6)
A111	12.7±2.8^{c}(56.2)	16.7±4.1^{c}(40.4)	2.0±1.8^{c}(25.0)	12.7±4.3^{bc}(38.8)
A2	16.0±2.8^{c}(70.8)	26.0±2.8^{b}(63.0)	2.6±1.5^{bc}(33.4)	17.3±2.8^{b}(53.0)
A22	10.7±2.8^{cd}(47.5)	12.0±3.8^{cd}(29.0)	1.3±1.8^{c}(16.7)	14.7±3.8^{b}(44.9)
A222	6.0±2.8^{d}(26.5)	8.7±3.8^{d}(21.0)	1.3±1.8^{c}(16.7)	6.0±2.8^{c}(18.4)

Survival of the nauplii after challenge with a lethal heat shock at 41 °C for 20 min (**A**) or with 10^7 cells/mL of *V. campbellii* (**B**). For each average, the respective standard deviation is added (mean ± S.D.). Values in the same column (for each strain) showing the same superscript letter are not significantly different ($p < 0.05$) (one-way ANOVA). Within each column, values between brackets express survival as a percentage of the control value.

GSL = Great Salt Lake. VC = Vinh Chau. A1 = 2h hydration + 24 h dehydration (1 cycle). A2 = 4 h hydration + 24 h dehydration (1 cycle), A11 = 2 h hydration + 24 h dehydration (2 cycles). A22 = 4 h hydration + 24 h dehydration (2 cycles), A111 = 2 h hydration + 24 h dehydration (3 cycles). A222 = 4 h hydration + 24 h dehydration (3 cycles). Control = without hydration/dehydration treatment.

4.3.3. Resistance against V. campbellii of nauplii hatched from cysts exposed to successive hydration/dehydration treatment

The survival of the GSL *Artemia* nauplii developed from cysts that had undergone 1 (A1) or 2 (A11) H/D cycles with 2 h hydration did not differ significantly from that of the control group, but was significantly higher than other treated groups (except for A11 and A2, which were not

significantly different) (Table 4.1 B). Increasing the number of H/D cycles to 3 (A111) or prolonging the hydration period from 2 h to 4 h (A2, A22, and A222) significantly reduced the survival of the *Vibrio*-challenged nauplii as compared to the control, with lowest survival being observed in the A22 and A222 groups. The survival of the *Artemia* nauplii belonging to the VC strain also exhibited a similar trend: the control, and the A1 and A11 *Artemia* nauplii did not differ significantly among each other. However, increasing the number of H/D cycles to 3 (A111 and A222 groups) significantly reduced the survival of the challenged nauplii ($P < 0.05$). In each treatment and in the controls, the GSL strain was more sensitive to the pathogen than VC.

4.4. Discussion

Embryos of *Artemia* develop ovoviviparously, yielding swimming nauplii upon release from females, or they develop oviparously, producing encysted gastrulae known as cysts (Liang and MacRae 1999; MacRae 2003). The *Artemia* cysts that enter into the diapause state, a physiological state of metabolic dormancy and enhanced stress resistance (MacRae 2010; Hand et al., 2011), are among the most resistant of all animal life history stages to environmental extremes (see review, Clegg et al., 1999). Hsps synthesized in diapause-destined embryos of *Artemia* have been reported to be a contributing factor to the high stress tolerance of *Artemia* embryos (King and MacRae 2012). In nature *Artemia* cysts normally encounter extreme environmental conditions when floating on the surface of their hypersaline environment and/or after being blown on the shore where they can be buried under masses of decaying biological matter (Clegg et al., 1999). In our study, by mimicking extreme environmental conditions for the cysts by exposing them to different H/D cycles, we aimed to determine the effect of these conditions on the cysts Hsp70 level, and further investigated if Hsp70 aids in yielding nauplii that are more stress-resistant after being

exposed to H/D cycles. Our results revealed that H/D cycles induced Hsp70 production in the *Artemia* cysts in a manner dependent on the number of H/D cycles and the duration of the hydration phase. In the GSL cysts, H/D treatment markedly increased the Hsp70 level, but the opposite was the case for the VC cysts. According to Clegg et al., (2000), the cysts produced in Vietnam are much more resistant to stress (*i.e.* high temperatures) than cysts produced in San Francisco Bay, suggesting that VC cysts have become adapted to higher temperatures. The difference between the two strains in the induction pattern of Hsp70 in response to H/D treatment could be associated with the difference in the prevailing environmental conditions in the two different geographical locations, from where these two strains originated. Our results also showed that after hatching, there was an apparent decrease in the level of Hsp70 in the emerged nauplii of both strains compared to the cysts. There is earlier evidence suggesting that the level of mRNAs encoding for Hsp70 and also for other molecular chaperones, such as p26, Hsp90 and Hsp110, appears to be lower in cyst-derived *Artemia* nauplii than in diapause-destined *Artemia* embryos (see review, MacRae 2010). The decrease was more prominent in the nauplii hatched from the treated cysts compared to the control cysts.

Previous studies have suggested that induction of Hsps, mainly Hsp70, is associated with the induction of resistance within an organism against both abiotic and pathogenic biotic stressors (Roberts et al., 2010; Baruah et al., 2012, 2014). In this study, we next determined whether alterations in the Hsp70 level in the cysts and in the emerged nauplii in response to H/D treatment of the cysts come with a downstream effect on the survival performance, measured in terms of resistance against thermal shock or *V. campbellii* challenge, of the emerged *Artemia* nauplii. Our results revealed that the GSL and VC *Artemia* nauplii emerging from the cysts exposed to a short period (2 h) of 1 or 2 cycles of hydration (*i.e.* A1 and A11 groups) had a thermotolerance similar

or higher than that of the control nauplii (emerged from untreated cysts). However, the stress tolerance was negatively affected by exposure to three H/D cycles. The decrease as a result of multiple H/D cycles was more prominent when a hydration period of 4 h was used, as compared with hydration of 2 h. This result suggests that irrespective of its other documented effects (see Chapter 3). H/D treatment can interact with the induction of thermotolerance and the up-regulation of Hsps. In fact a short period of hydration/dehydration exposure of cysts does not seem to cause a deleterious effect on the emerged nauplii of both strains. To further substantiate our findings, we determined the resistance of the *Artemia* nauplii emerged from the treated and control cysts towards pathogenic *V. campbellii*. Similar as for the observed thermotolerance results, exposure of cysts (for both strains) to H/D for a short period (2 h) of 1 or 2 cycles was shown to impose no adverse effect on the survival of *Vibrio*-challenged *Artemia* nauplii. This implies that a short period of H/D cycles does not prohibit starved *Artemia* nauplii to survive stress as caused by a thermal shock or by *Vibrio* exposure.

In conclusion, our results provide first experimental evidence that a limited exposure of cysts to H/D treatment (*i.e.* A1 and A11 groups) in GSL *Artemia* may lead to the induction of thermotolerance in the emerged nauplii whereas a more pronounced H/D (A111, A2, A22, A222) treatment results in the opposite effect. However, all GSL cysts being exposed to H/D cycles showed an upregulation of Hsp70. The results reported in this study augment our current understanding of stress responses in aquatic invertebrates. The impact of H/D cycles associated with stress response should be taken into consideration especially when using *Artemia* nauplii emerged from cysts with unknown history as a model organism for future research and applications.

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Chapter 5

Nutrition can enhance protection against biotic stressors in *Artemia* nauplii hatching from cysts exposed to hydration/dehydration cycles

Abstract

The present study aimed at elucidating the effects of two baker's yeast types, *i.e.* wild-type (WT) and its mutant Mnn9, in combination with autoclaved *Aeromonas hydrophila* bacterial strain LVS3 on the performance of *Artemia franciscana* nauplii in gnotobiotic conditions, hatched from cysts which had been exposed previously to different H/D cycles, and using survival and growth as criterion. The resistance to challenge with *Vibrio campbellii* was also examined.

Our results revealed that the survival of starved nauplii, hatching from cysts exposed to H/D cycles, was poor and that they were not protected against pathogenic *Vibrio*. In combination with dead bacteria, live yeast exerted a strong positive effect on *Artemia* survival when it was provided as sole diet following exposure of nauplii to the pathogen. However, the effects on *Artemia* nauplii emerged from cysts exposed to a long period of H/D treatment were too severe to be addressed by supplying any of the diets consisting of yeast and bacteria.

This study suggests that suitable feeding in terms of the digestibility, size and nutrients content (*i.e.* yeast cell wall mutants and probiotic bacteria) provided to stressed *Artemia* nauplii, emerged from cysts exposed to limited H/D treatments, provides improved protection against deleterious effects. However, in the most extremely stressed larval *Artemia,* less improvement may occur. These results can be useful as a potential method for improving survival of *Artemia* nauplii after it has been exposed to different extreme environmental (abiotic and biotic) stressors.

5.1. Introduction

Nauplii of the brine shrimp *Artemia* are the most commonly used live food in aquatic larviculture around the globe. Due to their nutritional value and size, *Artemia* nauplii, either freshly hatched or after nutritional enrichment, are a suitable substitute for the zooplankton that is the natural food of early-stage fish and crustacean larvae (Sorgeloos et al., 2001). Different techniques have been developed to enhance the nutritional profiles of nutritionally deficient *Artemia* strains (*i.e.* lipids, fatty acids, amino acids, enzymes, minerals and vitamins). Also new feeds and supplements are being developed to reduce stress and mortality, maintain the health of cultured organisms, and to stimulate the mechanisms of non-specific defense against diseases (Marques et al., 2006a). Among such nutritional components, tested on newly hatched *Artemia* nauplii, are β-glucans (Sung et al., 1996; Sritunyalucksana et al., 1999; Wang and Chen, 2005), mannoproteins (Tizard et al., 1989), lipopolysaccharides (Takahashi et al., 2000), peptidoglycans (Itami et al., 1998; Boonyaratpalin et al., 1995), baker's yeast (Coutteau et al., 1990), live bacteria (Intriago and Jones, 1993; Marques et al., 2005) and dead bacteria (Alabi et al., 1999; Keith et al., 1992; Vici et al., 2000; Marques et al., 2005).

For this purpose, the development and validation of the *Artemia* gnotobiotic test system to study host-microbial interaction in the presence of selected microbiota (Marques et al., 2004a, b) has been proven very useful. In experimental studies, baker's yeast (*Saccharomyces cerevisiae*) has been found to be an excellent source of β-glucans and chitin. These compounds, together with mannoproteins, are major constituents of the yeast cell wall (Magnelli et al., 2002). Also the presence of bacteria in the culture medium has been proven to improve growth performance of aquatic animals in general (Intriago and Jones, 1993; Marques et al., 2004a). These new components are interesting feeds for *Artemia* since they have a small particle size, a high protein content, good buoyancy in the water column and low production costs (Coutteau et al., 1990).

A common trait of all these feeds is the fact that they are utilized under xenic conditions; this implies that in most cases there is no way to distinguish between the nutritional contribution of the bacteria associated with certain *Artemia* feeds, and of the feed itself (Douillet, 1987). Only under axenic conditions can the real value of a feed be appreciated. The nutritional value of *Artemia* from different geographical sources may be variable and environmental conditions in the natural habitats and during post-harvest processing, under which *Artemia* cysts may pass through several cycles of hydration/dehydration (H/D), may change. This can severely impair the quality of cysts and also the quality and performance of the emergent larvae (Vanhaecke and Sorgeloos, 1982; Lavens and Sorgeloos, 1987). In Chapter 3, we showed the negative effect (*i.e.* decreased cyst hatching, reduced starved naupliar longevity and drop in individual energy content, loss in vitamin C and fatty acid content) of subjecting cysts to several H/D cycles. The nutritional status of *Artemia* may also be reflected in reduced resistance against pathogens. In the present study, we thus aimed to investigate whether a diet composed of yeast and bacteria has any beneficial effect for *Artemia* nauplii (*i.e.* improved survival, growth and resistance against pathogens), hatching from cysts previously exposed to H/D cycles.

For this purpose, we used wild-type (WT) *Saccharomyces cerevisiae* and its mutant Mnn9, the cell wall of which has reduced mannose and increased glucan content as compared to wild type yeast, which is less digestible compared to the mutant one. For bacteria we used autoclaved *Aeromonas hydrophila* strain LVS3. The performance of *Artemia* in terms of survival and growth was used as criterion to assess the positive effect of the feed on *Artemia*. Both yeast and bacteria were offered to *Artemia* nauplii as food source in a gnotobiotic *Artemia* culture system (Marques et al., 2006c, d), which has proven useful in various stress response studies, especially in establishing cause-effect relationship of stress-inducing agents (Marques et al., 2006a, b; Sung et al., 2007; Baruah et al., 2014). Such system permits eliminating the interference of multiple factors (such as changing microbial communities, rearing conditions)

during the experimental period and eventually allows determining the biological responses of the host towards the specific testing agent (Marques et al., 2006a).

5.2. Material and methods

5.2.1. Cyst samples

Experiments were performed with two strains of *A. franciscana*, both harvested in 2007, and originating from two different geographical locations, where they experience different environmental factors: one commercial dry sample originating from Great Salt Lake (GSL), Utah, USA (INVE Aquaculture Belgium, Type EG (batch number: 21425), and a second sample from Vinh Chau (VC) salt fields, Vietnam (ARC code 1718), supplied by Can Tho University, Vietnam. Both samples had been stored at + 4 °C since their arrival at the Laboratory of Aquaculture & *Artemia* Reference Center, which is the current procedure to ensure maximal viability and hatchability of the cysts, even in dried form (Lavens and Sorgeloos, 1996).

5.2.2. Experimental design

This study comprised three experiments and their experimental design was as follows: in a first experiment, we aimed to determine the effect of pathogenic bacteria on survival of starved *Artemia* nauplii hatched out from cysts that had been subjected to several hydration/dehydration cycles. Hatched nauplii that developed into instar II within the next 4 – 6 h (nauplii's mouth opens, allowing ingestion of *Vibrio*) were used in the experiments. The nauplii were hatched under axenic conditions and subsequently challenged with live *Vibrio campbellii* at a density of 10^7 cells mL^{-1}. Nauplii were not fed throughout the two days of the experiment and *Vibrio* was supplied at time zero (at the start of the experiment).

In a second experiment, we aimed to determine the effect of feeding baker's yeast on individual length and survival of *Artemia* nauplii (the same type of nauplii as used in experiment 1). The two live and axenic yeast strains (WT and Mnn9) were harvested in the exponential growth phase and were used as major feed for the *Artemia* nauplii in combination with dead LVS3 (as a supplemented part of the feed). The feed regime was fixed at 1×10^4 cells mL^{-1} for WT or Mnn9 and supplemented with autoclaved *A. hydrophila* strain LVS3 at 1×10^7 cells mL^{-1} as food. Nauplii were fed only once throughout the two days of the experiment and feed was supplied at time zero.

In a third experiment, we determined the effect of *V. campbellii* on survival of *Artemia* nauplii (the same type of nauplii as used in experiment 1). In this experiment the axenically hatched *Artemia* nauplii were fed with the same two different types of baker's yeast in combination with dead LVS3, using the same feed regime as in experiment 2, by using yeast and bacteria harvested in the exponential growth phase (the best results were obtained with this growth phase in experiment 2). The *Artemia* nauplii were subsequently challenged with live *Vibrio campbellii* at a density of 10^7 cells mL^{-1} added 12 h after feeding *Artemia.* For that purpose, in a laminar flow hood, the pathogen was provided to each one of the 4 replicates.

In each experiment after 12, 24, 36 and 48 h of culture, the number of swimming larvae was determined and survival percentage was calculated. Each treatment in every experiment was carried out in quadruplicate.

5.2.3. Hydration/dehydration cycles

As in Chapter 3, cysts used for these experiments were exposed to successive hydration/dehydration (H/D) cycles by incubating 1.6 g of cysts of each strain in a 1-litre cylindroconical glass cone containing 800 mL of medium, *i.e.* fresh water (tap water) for the

hydration step, NaCl-saturated brine (280 – 300 g L^{-1}) for the dehydration step, at 28 °C under strong aeration. A first group of three cones was set up; the cysts in the first cone were exposed to one H/D cycle (2 h hydration, 24 h dehydration), the second one to two cycles, and the third one to three (named A1, A11 and A111, respectively). In parallel, for each strain three other cones went through a similar set-up, but with each hydration period lasting for 4 h (the corresponding treatments named A2, A22 and A222). The above operation was repeated nine times for each treatment, in order to produce sufficient cyst material for the subsequent analyses, and the samples, corresponding to the same treatment out of six, were pooled for storage and use. After the H/D steps, a fraction of each cyst sample was directly used in the experiments and the remaining fractions were immediately stored in + 4 °C in NaCl-saturated brine (280 – 300 g L^{-1}) for further analysis. *Artemia* nauplii emerged from cysts that were not exposed to H/D cycles served as controls.

5.2.4. Axenic culture of yeast

To examine the nutritional value and verify the digestibility of live baker's yeast (*S. cerevisiae*) by *Artemia*, one null-mutant of yeast (Mnn9) (isogenic deletion strain derived from baker's yeast strain BY 4741) and the wild type strain (WT) were fed to *Artemia*. The former strain is defective in the synthesis of mannoproteins in the outer cell wall and has proven to be a suitable food for *Artemia*, whereas the wild type strain is poorly digestible. Both strains have been frequently investigated for their nutritional and immunostimulatory properties through gnotobiotic *Artemia* tests (Marques et al. 2004 a, b, Marques et al. 2006 a, b). Yeast cultures were performed according to procedures described previously by Marques et al. (2004 a, b), using minimal Yeast Nitrogen Base culture medium (YNB). Yeast was harvested by centrifugation (± 800 × g for 10 min) in the exponential growth phase (after 20 h). Yeast cell

concentrations were determined with a Bürker haemocytometer. Yeast suspensions were stored at 4 °C until the end of each experiment (maximum storage of one week).

5.2.5. Bacterial strains and growth conditions

Two bacterial strains were selected, *i.e.* firstly *Aeromonas hydrophila* strain LVS3, which is commonly used as feed at sub-optimal feeding levels for *Artemia* nauplii in *Artemia* challenge tests (Defoirdt, et al., 2005). According to the standard procedure *Aeromonas hydrophila* was used as food as autoclaved bacteria in order to eliminate the possibility of microbial interference with the yeast and because its properties as a food source in gnotobiotic *Artemia* tests have been well described (Defoirdt et al., 2007; Marques et al., 2005; Verschuere et al., 1999, 2000). *Vibrio campbellii* strain LMG21363 was used for its pathogenic effect towards *Artemia* and shrimp (Soto-Rodriguez et al., 2003; Gomez-Gil et al., 2004; Marques et al., 2005; Defoirdt et al., 2007). The two bacterial strains were cultured and harvested according to procedures previously described by Marques et al. (2005). Pure cultures of the two bacterial strains were obtained from the Laboratory of Microbial Ecology and Technology and from the Laboratory of Microbiology, both at Ghent University, Belgium. The bacterial strains were stored at -80 °C since their arrival at the Laboratory of Aquaculture & *Artemia* Reference Center (ARC), Ghent University, Belgium and grown overnight at 28 °C on marine agar, containing Difco ™ marine broth 2216 (37.4 g L^{-1}, BD Biosciences) and agar bacteriological grade (20 g L^{-1}, ICN). For each bacterial strain a single colony was selected from the plate and incubated overnight at 28 °C in 50 mL Difco ™ marine broth 2216 on a shaker (150 rpm) for 24 h. Exponential-grown bacteria were harvested by centrifugation (15 min; ± 2200 × g), the supernatant was discarded and the pellet was resuspended in 20 mL filtered autoclaved sea water (FASW). Bacterial densities were determined by spectrophotometry (OD_{550}), assuming that an optical density of 1.00 corresponds to 1.2×10^9 cells mL^{-1}, according to McFarland

standard (BioMerieux, Marcy L'Etoile, France). After cultivation, the pathogen was immediately used, while the LVS3 live bacterial suspensions were stored at +4 °C until the end of each experiment. The LVS3 suspension was autoclaved prior to feeding.

5.2.6. Artemia gnotobiotic cultures

Bacteria-free cysts and nauplii were obtained using the procedure described by Marques et al. (2004b). A few grams from each treatment of hydrated/dehydrated cysts were hydrated in 90 mL tap water for 1 h with strong aeration in non-axenic conditions. The recipient with the cysts was then transferred to a laminar flow hood, where decapsulation was performed using autoclaved and sterile tools. A 0.22 µm-filtered aeration was provided to avoid bacterial contamination. Then, 50 mL of cold sodium hypochlorite (NaOCl) containing 15 % (w/v) active chlorine and 3.3 mL of 32 % (w/v) sodium hydroxide (NaOH) were added to the hydrated cysts. The reaction was stopped after 150 s by adding 70 mL of sterile sodium thiosulphate pentahydrate ($Na_2S_2O_3.5H_2O$) (10 mg L^{-1}). Decapsulated cysts were washed several times carefully with filtered autoclaved sea water (FASW) and collected over a 50-µm sterile sieve. A few mg of these cysts were then transferred to separate, sterile 50-mL falcon tubes (four replicates per H/D treatment) containing 30 mL of FASW and capped. For hatching incubation, the tubes were placed on a rotor at 4 cycles' min^{-1} to prevent clogging and sedimentation of the cysts. Cysts were kept at 28.0 ± 0.5 °C and exposed to constant incandescent light (± 41µE m^{-2} s^{-1}) for 18 - 24 h. Consequently, hatched nauplii that developed into instar II within the next 4 - 6 h were used in the experiments (only in instar II, the nauplii's mouth opens, allowing ingestion of *Vibrio,* yeast or/and bacteria). Twenty nauplii (instar II) were picked and transferred to Falcon tubes containing 30 mL of FASW. Nauplii were fed with WT-yeast or Mnn9-yeast, in combination with dead LVS3 at 1×10^7 cells dead LVS3 mL^{-1}) plus 1×10^4 cells yeast mL^{-1} and challenged with *V. campbellii* at a density of 10^7 cells mL^{-}

[1]. Each treatment consisted of four Falcon tubes (replicates). Falcon tubes were placed on a rod rotating at 4 cycles min^{-1}, exposed to constant incandescent light (± 41µE m^{-2} s^{-1}) at 28 ºC. At the end of every 12 h during 2 days, the number of swimming larvae was determined and survival percentage was calculated. All manipulations were done under a laminar flow hood in order to maintain sterility of the cysts and nauplii.

5.2.7. Method used to verify axenity

Axenity of feed, decapsulated cysts and *Artemia* cultures was checked at the end of each experiment using a plating of marine agar (MA) following the procedure described by Marques et al. (2004a,b). In challenge treatments, the axenity of *Artemia* culture was always checked before challenge using the same methods. Contaminated cultures tubes were not considered for further analysis and in that case the treatment was repeated. Dead LVS3 was provided to *Artemia* as food using aliquots of autoclaved concentrated bacteria (autoclaving at 120 °C for 20 min). After autoclaving, bacteria were plated to check if they were effectively killed by this method. For this purpose, 100 µL of the culture medium were transferred to marine agar (MA; n = 3), containing Difco ™ marine broth 2216 (BD Biosciences, 3.74 % w/v) and agar bacteriological grade (ICN, 2 % w/v). Absence of bacterial growth was monitored after incubating plates for 5 days at 28 °C. The autoclaving treatment was 100 % effective, since no bacterial growth was observed on the MA after 5 days of incubation.

5.2.8. Survival and growth of Artemia

Survival and growth of *Artemia* nauplii were determined according to procedures described by Marques et al. (2004a, b). During each experiment 1, 2 and 3 (experimental design see section 5.2.2) the number of swimming larvae was determined every 12 h and survival percentage was calculated as $N_t \times 100 / N_o$ where N_t and N_o are final and initial numbers of larvae, respectively.

At the end of experiment 2 (day 2 after the feeding test), living larvae were fixed with Lugol's solution allowing to measure their individual length, using a dissecting microscope equipped with a drawing mirror, a digital plan measure and the software *Artemia* 1.0® (Marnix Van Damme).

5.2.9. Statistical analysis

Values of larval survival percentages were arcsin-square-root transformed to render the data normal and hence suitable to the linear modelling framework (Warton and Hui, 2012) prior to performing statistical analysis. For each strain, each different type of feed and challenge type (non-challenge versus challenge with pathogenic bacteria) within each experiment, the data of larval survival for 12, 24, 36 and 48 h were subjected to a linear mixed model using SAS version 9.4 (SAS Institute, Cary, NC) (Verbeke and Molenberghs, 2000). To detect whether the observed variations in mean survival % were statistically significant or not, a $P < 0.05$ was considered as statistically significant. Survival % was used as the dependent variable while time, H/D treatment, types of feed (WT and Mnn9) and challenge versus non-challenge were used as independent variables. Interaction effects between time, H/D treatment, type of feed and challenge were assessed. All interaction effects that were not significant were dropped from the model and pairwise comparison between the different H/D treatments was done using the Tukey adjustment method for multiple testing which offers adjusted *P*-values (Sherri, 2012). Comparison of variations in mean survival % over time was done for non-challenged (starved) nauplii versus nauplii starved and challenged with pathogenic bacteria (Experiment 1), between nauplii fed with WT and those fed with Mnn9 (Experiment 2), and between nauplii fed with WT versus Mnn9, both challenged with pathogenic bacteria (Experiment 3). Line plots were generated for the non-transformed mean survival percentage (over 4 replicates) using R Software (Version 3.2.2, R Core Team, 2017).

Finally, individual length data, obtained in Experiment 2, were square root transformed before further statistical analysis. For each strain and each type of feed (WT and Mnn9), the data of larval length at 48 h were subjected to one-way ANOVA to detect an effect between the different H/D treatments. For the one-way ANOVA test (SPSS, version 16.0), Tukey test was used to detect significant differences between the experimental sample means, and $P < 0.05$ was considered as significant.

5.3. Results

5.3.1. Challenge test of starved nauplii

In experiment 1, the mean survival % of all starved nauplii of the two strains challenged with live *V. campbellii* was substantially lower than that of the non-challenged starved ones (Fig. 5.1, $P < 0.05$) in each H/D treatment and each time of incubation. In the case of the GSL strain, the survival of the non-challenged starved nauplii emerged from the cysts that were exposed to 2 h of repeated H/D and the control (nauplii emerging from cysts not exposed to H/D cycles) presented higher survival compared to all other nauplii that emerged from the cysts that were exposed to 2 h of repeated H/D and challenged or for those exposed to 4 h of repeated H/D and non-challenged or challenged with *Vibrio*. For the VC strain, the survival of the challenged and non-challenged *Artemia* nauplii also exhibited almost a similar trend as in the GSL strain (Fig. 5.1). Through the Least Squares Means (LSM) Tukey pairwise comparison (results not shown), for both strains a hydration period of 2 h for 1 cycle (A1) did not affect the survival of challenged *Artemia* as compared to nauplii from non-H/D exposed cysts. However, increasing the number of H/D to 3 cycles (A111) and/or the hydration period from 2 h to 4 h (A22 and A222) significantly affected the survival of the *Vibrio*-challenged nauplii in a negative way, with lowest survival being observed in A111 and A222.

The results also revealed that the three-factors interaction effect between treatment (H/D cycles), time (12, 24, 36 and 48 h) and challenge (starved nauplii, non-challenged and challenged with *Vibrio*) was not statistically significant (F (6, 154) = 2.09, *P*-value = 0.0569) and (F (6, 154) = 0.77, *P*-value = 0.5951) for GSL and VC strains, respectively (Table 5.1). Moreover, for GSL there was a non-significant interaction ($P > 0.05$) between H/D treatment and challenge meaning that the effect of the challenge on the survival of nauplii does not depend on the treatment. However, other pairwise interactions were highly statistically significant ($P < 0.0001$) (Table 5.1). For VC, on the other hand, all pairwise interactions were statistically significant ($P < 0.05$). This indicates that the survival of VC nauplii varies over time according to the different treatments and depending on whether or not the starved nauplii were challenged with *Vibrio* (Table 5.1). The overall performance of the challenged and non-challenged starved nauplii was better in the VC strain than in GSL (Fig. 5.1), as in our previous study (Chapter 3). As the energy content in the cysts dropped, the nauplii hatching from these cysts showed lower survival and thus less resistance against pathogenic bacteria.

Table 5.1: Experiment 1: Statistical results of tests for fixed effects (linear mixed model) of H/D treatment, time and challenge with *Vibrio campbellii*, and their interactions, on the survival of nauplii. Num.DF = numerator degrees of freedom; Den.DF = denominator degrees of freedom.

Tests of fixed effects								
Strain	**GSL**				**VC**			
Effect	**Num. DF**	**Den. DF**	**F-value**	**P-value**	**Num. DF**	**Den. DF**	**F-value**	**P-value**
H/D Treatment	6	42	39.9	<.0001	6	42	27.5	<.0001
Challenge	1	42	457.3	<.0001	1	42	217.6	<.0001
H/D Treatment*Challenge	6	42	1.7	0.1557	6	42	3.7	0.0046
Time	1	154	1397.7	<.0001	1	154	2051.1	<.0001
Time*H/D Treatment	6	154	11.2	<.0001	6	154	2.4	0.0320
Time*Challenge	1	154	44.9	<.0001	1	154	43.9	<.0001
Time*H/D Treatment*Challenge	6	154	2.1	0.0569	6	154	0.8	0.5951

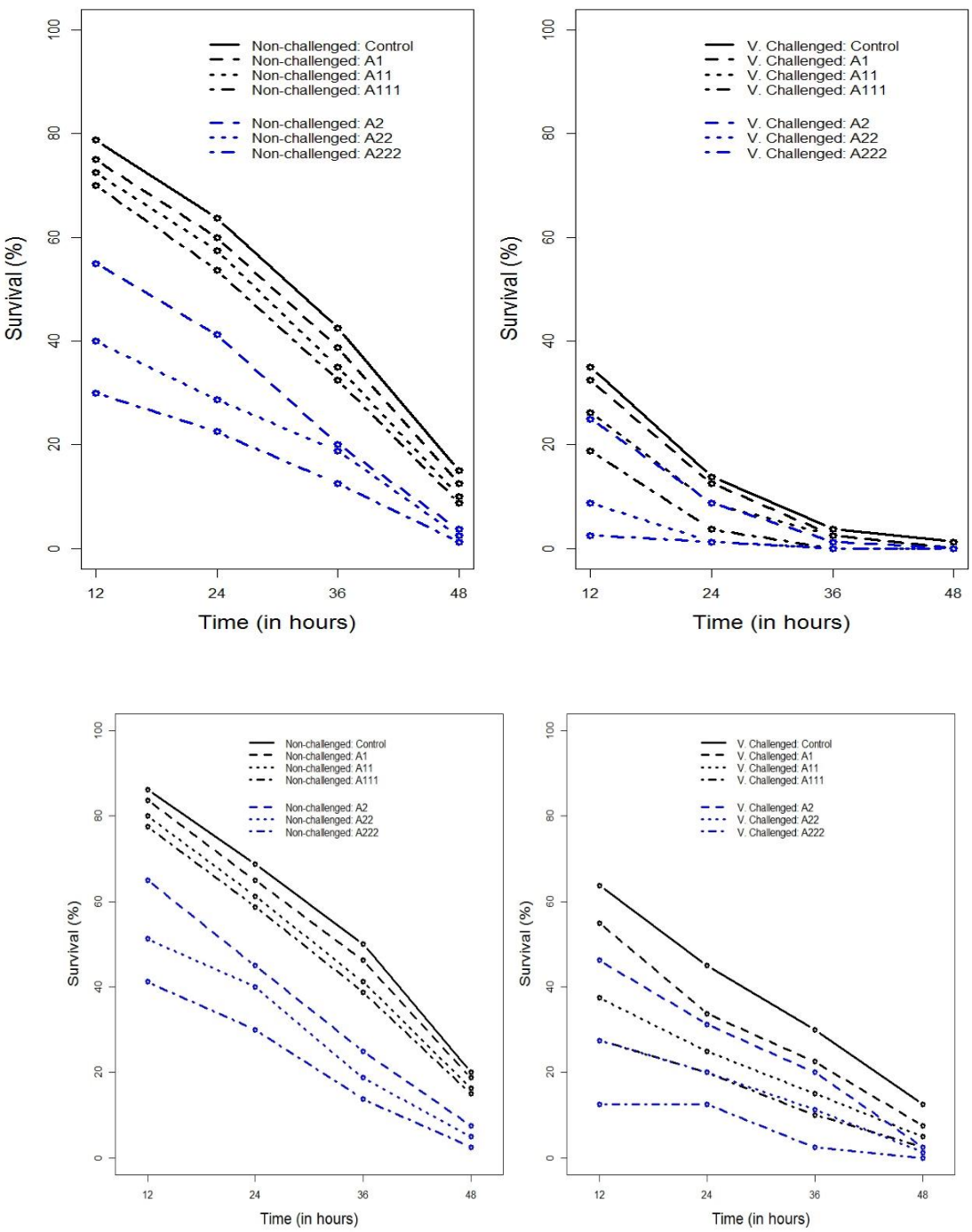


Figure 5.1: Line plot of survival (%) over a 48 h period of starved non-challenged (left) and starved challenged (right) newly hatched nauplii of GSL (top) and VC (below) cysts previously exposed to various H/D treatments (A1, A11, A111, A2, A22 and A222) at different time intervals (12, 24, 36 and 48 h). For abbreviations of H/D treatments, see Table 3.1.

5.3.2. Artemia performance fed live yeast cells

In experiment 2, to determine the effect of feeding, in terms of growth and survival of nauplii hatched axenically from cysts that had undergone different H/D cycles, *Artemia* nauplii were fed with one isogenic mutant strain (Mnn9) of baker's yeast (*Saccharomyces cerevisiae*) and compared with nauplii fed wild type (WT) yeast, in combination with the reference autoclaved bacterial strain LVS3 under gnotobiotic conditions. For both *Artemia* strains, the LSM Tukey pairwise comparison showed that at each time of incubation the mean survival of nauplii fed with either type of yeast, developed from cysts that had undergone 1 or 2 cycles of 2 h (A1 and A11, respectively) or 1 cycle of 4 h (A2), did not differ significantly ($P > 0.05$) from that of the nauplii hatching from the control (non-H/D treated cysts), except in the case of A11 for GSL, and the case of A11, A2 for VC after 12 h. However, increased H/D cycles (A22 and A111) had a significantly negative effect on the survival of the nauplii, with the lowest survival being observed in A222 for the use of the two baker's yeasts (results of the pairwise comparison not shown). Pairwise comparison in both *Artemia* strains also showed that mean survival of fed nauplii was not significantly different ($P > 0.05$) when the WT and the isogenic yeast mutant strain (Mnn9) were used as feed, except in the case of A2 at 24 and 36 h and A22 at 48 h for GSL, and A11 at 24 h and A222 at 12 h for the VC strain. In both strains the use of the two types of baker's yeast, in case of non-challenge, generally resulted in considerably higher *Artemia* performance in terms of survival (Fig. 5.2), than in the starved non-challenged nauplii (Fig. 5.1). Also differences in survival were observed between starved non-challenged nauplii hatched from non-H/D treated cysts (Fig. 5.1) (experiment 1) and non-challenged nauplii hatched from non-H/D treated cysts in the test fed with WT and Mnn9 (experiment 2) (Fig. 5.2).

In addition, the linear mixed model revealed that the three-factors interaction effect between treatment (H/D cycles), time (12, 24, 36 and 48 h) and type of feed (WT and Mnn9) was not

statistically significant (F (6, 154) = 0.16, *P*-value = 0.9876) and (F (6, 154) = 0.27, *P*-value = 0.9485) for the GSL and VC strain, respectively (Table 5.2). Moreover, for both strains there was a non-significant interaction ($P > 0.05$) between H/D treatments and types of feed and also between time and types of feed. However, for each strain the pairwise interaction between time and H/D treatment was highly statistically significant (Table 5.2, *P*-value < 0.0001).

Table 5.2: Experiment 2: Statistics of tests for fixed effects (linear mixed model) of H/D treatment, time and type of feed, and their interactions on the survival of nauplii. Types of feed = WT-yeast and Mnn9-yeast. Num.DF = numerator degrees of freedom; Den.DF = denominator degrees of freedom).

Tests of fixed effects								
Strain	**GSL**				**VC**			
Effect	**Num. DF**	**Den. DF**	**F-value**	**P-value**	**Num. DF**	**Den. DF**	**F-value**	**P-value**
H/D Treatment	6	42	23.5	<.0001	6	42	28.7	<.0001
Type of feed	1	42	9.0	0.0045	1	42	8.7	0.0051
H/D Treatment*Type of feed	6	42	0.1	0.9930	6	42	0.4	0.8453
Time	1	154	605.4	<.0001	1	154	539.9	<.0001
Time*H/D Treatment	6	154	7.0	<.0001	6	154	6.9	<.0001
Time*Type of feed	1	154	0.5	0.4923	1	154	0.5	0.4832
Time*H/D Treatment*Type of feed	6	154	0.2	0.9876	6	154	0.3	0.9485

Furthermore, in all cases, feeding the mutant-yeast strain (Mnn9) supported the best growth of nauplii, as larval length was significantly higher in the non-H/D treated nauplii and in A1 and A11 larvae compared to A22 and A222 larvae (Table 5.3; one-way ANOVA, $P > 0.05$).

Table 5.3: Average individual length (mm) after 48 h of *Artemia* nauplii, hatched from cysts previously exposed to different H/D treatments, and fed with live yeast cells (strains WT and Mnn9) and supplemented with dead LVS3.

Strain	**GSL**		**VC**	
Treatment	**Dead LVS3+WT**	**Dead LVS3+ Mnn9**	**Dead LVS3+WT**	**Dead LVS3+ Mnn9**
[1]Control	1.2 ± 0.1^{a}	1.3 ± 0.2^{a}	1.0 ± 0.1^{a}	1.2 ± 0.1^{a}
A1	1.1 ± 0.2^{a}	1.3 ± 0.1^{a}	1.0 ± 0.1^{a}	1.1 ± 0.1^{a}
A11	1.0 ± 0.1^{a}	1.1 ± 0.1^{ab}	1.0 ± 0.1^{a}	1.0 ± 0.1^{ab}
A111	0.9 ± 0.1^{ab}	1.0 ± 0.1^{b}	0.8 ± 0.1^{b}	0.9 ± 0.2^{b}
A2	1.0 ± 0.2^{a}	1.1 ± 0.2^{ab}	1.0 ± 0.1^{a}	1.0 ± 0.1^{ab}
A22	0.8 ± 0.1^{b}	1.0 ± 0.1^{b}	0.8 ± 0.1^{b}	0.9 ± 0.2^{b}
A222	0.8 ± 0.1^{b}	0.9 ± 0.2^{b}	0.8 ± 0.1^{b}	0.8 ± 0.1^{b}

For each strain and each type of feed, superscripts in each column show significant difference between different hydration/dehydration treatments (one-way ANOVA). Data are mean (n=4) ± standard deviation. [1]Control = not exposed to H/D. Significance level was set at $P < 0.05$. For abbreviations, see Table 3.1.

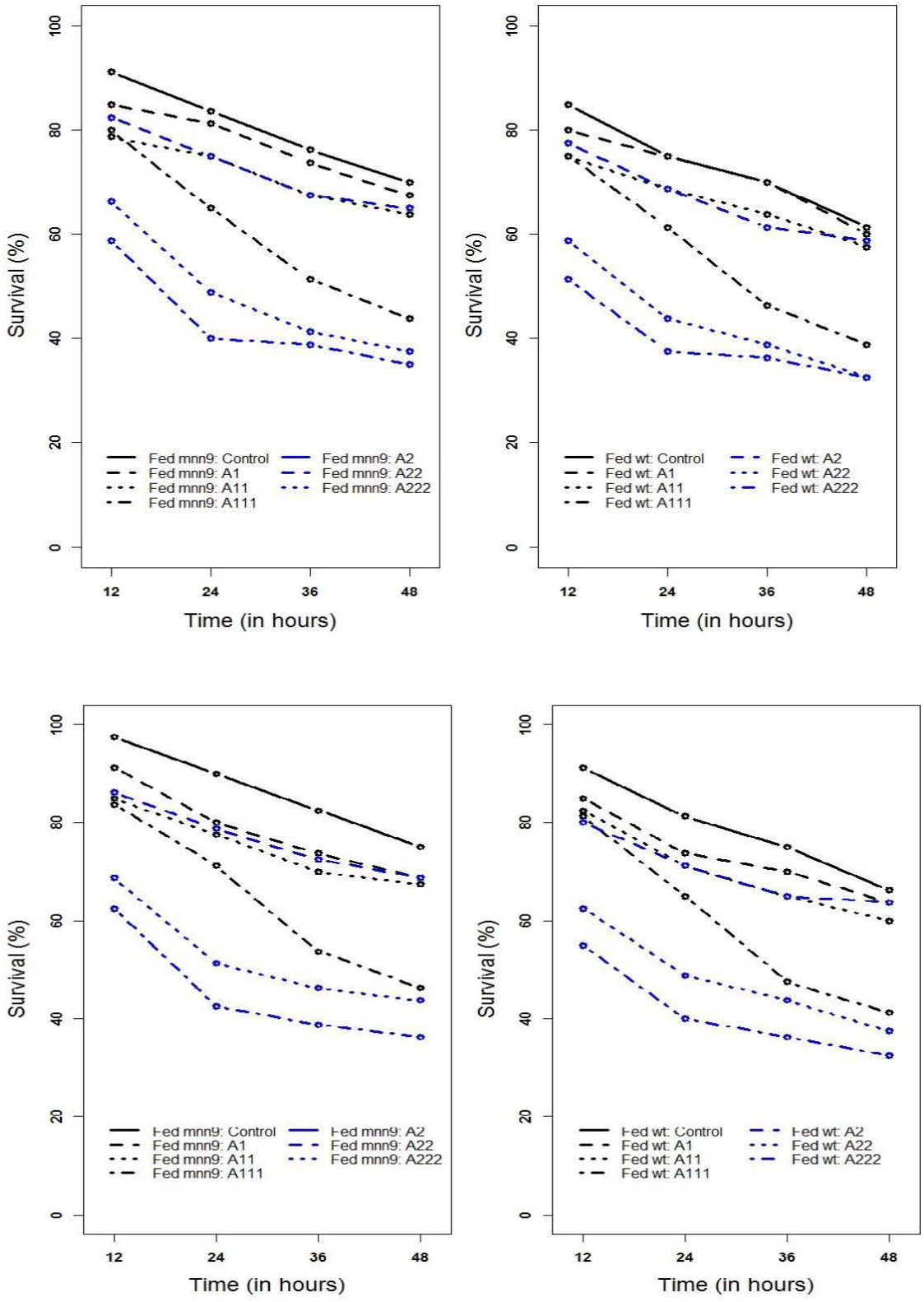


Figure 5.2: Line plot of survival (%) over a 48 h period of nauplii from **GSL** (top) and **VC** (below) cysts previously exposed to various H/D treatments (A1, A11, A111, A2, A22 and A222) at different time intervals (12, 24, 36 and 48 h). The nauplii were fed with yeast cells (strains Wt and Mnn9) and supplemented with dead LVS3. For abbreviations of H/D treatments, see Table 3.1.

5.3.3. Artemia performance fed LVS3 bacteria and live yeast cells and challenged with V. campbellii

Artemia nauplii were fed with one isogenic mutant strain of baker's yeast (*S. cerevisiae*) and compared with nauplii fed WT yeast under gnotobiotic conditions and challenged with a live pathogen bacterium. The survival of challenged larvae of both *Artemia* strains hatched out from cysts exposed to 3 H/D cycles exhibited substantially lower survival as compared to the challenged nauplii hatching from non-H/D exposed cysts, while also a reduced survival occurred for the treatment with 1 H/D cycle and with a moderately reduced survival for the treatment with 2 H/D cycles (Fig. 5.3). In all treatments, nauplii fed with yeast cells (WT or Mnn9) in combination with dead LVS3 and then challenged with *Vibrio*, survived until 2 days, but with lower survival, ranging from zero with WT yeast and 1.3 to 2.5 % with Mnn9 for A222 in both *Artemia* strains, compared to the survival of the nauplii hatching from non-H/D treated cysts, 16.3 % of which survived until 48 h with WT yeast and 26.3 % with Mnn9 in case of GSL, and 27.5 % (WT yeast) and 35.0 % (Mnn9) for the VC strain (Fig. 5.3). In all cases, the Mnn9 yeast provided a higher protection against the pathogen than WT after 24 h of culture, but with higher mortality at the end of the 48 h observation period (Fig. 5.3).

The results of the LSM Tukey pairwise comparisons at each time of incubation obtained in experiment 3 for both strains showed that the mean survival of the challenged nauplii fed with WT was in most cases significantly lower than those fed with the Mnn9 strain (results of pair wise comparison not shown), illustrating the effect of the type of feed.

Moreover, the three-factor interaction effect between treatment (H/D cycles), time (12, 24, 36 and 48 h) and type of feed in case of *Vibrio* challenge (WT + *Vibrio* and Mnn9 + *Vibrio*) was not statistically significant ($F(6, 154) = 0.30$, P-value = 0.9357) and ($F(6, 154) = 0.19$, P-value = 0.9780) for the GSL and VC strain, respectively (Table 5.4). However, the pairwise

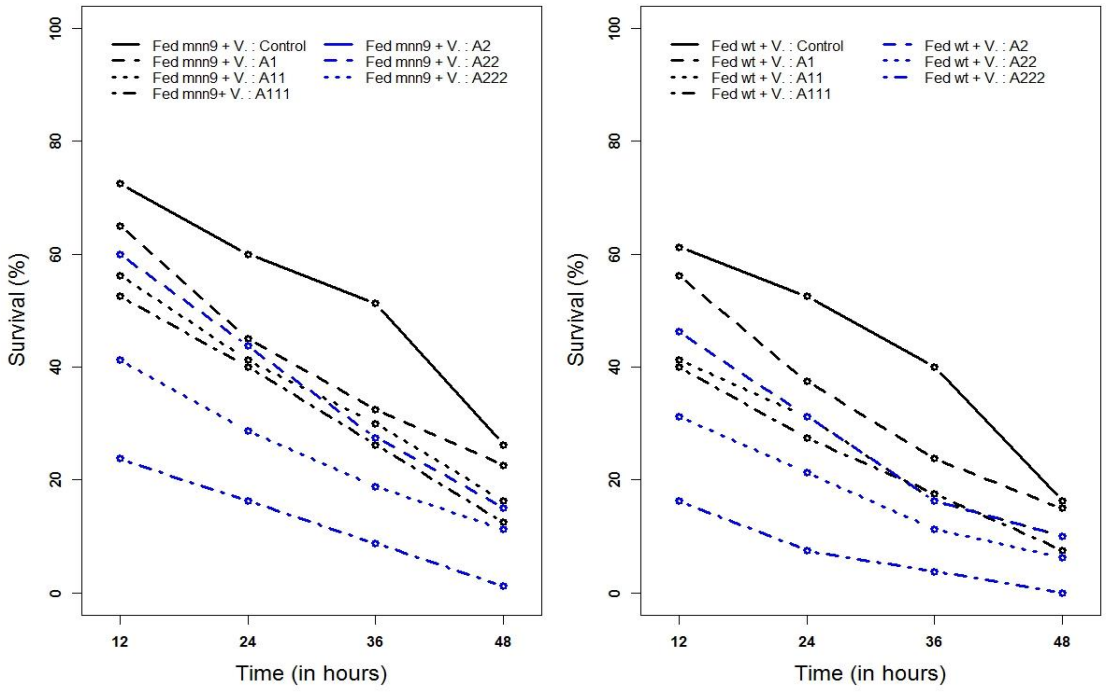
interaction between H/D treatment and time was statistically significant for both strains (Table 5.4, $P < 0.05$). This means that, keeping time fixed, the survival % was found to be significantly different for at least one H/D treatment pair combination (Tukey adjusted *P*-value < 0.05). On the other hand, keeping H/D treatments fixed, the expected survival % was observed to vary significantly across time. Other interactions were not statistically significant (Table 5.4, *P*-value > 0.05).

On the other hand, the challenge with pathogenic bacteria showed that for each strain, the survival of the challenged nauplii when fed (experiment 3) was significantly higher than when starved challenged (experiment 1) and the survival of the control nauplii increased using the two types of feed from 3.8 to 4.3 times with WT and Mnn9 respectively. While for A222 the survival even increased from 5.7 to 12.5 times with WT and Mnn9 respectively, in the first 24 h (Fig. 5.1 and 5.3). This suggests that the use of live yeast cells as feed in combination with dead bacteria has a positive effect on survival and protects the nauplii against pathogenic bacteria. Moreover, comparison of the resistance of the two *Artemia* strains against pathogenic *Vibrio* showed that the GSL strain is more sensitive to the pathogenic effect of *V. campbellii* than its VC counterpart (Fig. 5.1 and 5.3).

Overall, our results revealed that the performance of challenged *Artemia* strains in terms of survival when exposed to a biotic stressor was significantly higher in nauplii fed with live yeast cells (Mnn9) and supplemented with dead LVS3 compared to the use of WT yeast. However, the fed nauplii emerged from cysts exposed to multiple H/D cycles (*e.g.* A222) and challenged with pathogenic bacteria, showed a lower growth and survival as compared to the non-H/D treated control, which cannot be addressed through nutrition. The state of these nauplii cannot be linked only to the effect of the *Vibrio* challenge but also to the deleterious effect of successive H/D cycles.

Table 5.4: Experiment 3: Statistics of tests for fixed effects (linear mixed model) of H/D treatment, time and type of feed when challenged with *Vibrio*, and their interactions on the survival of nauplii. Type of feed + V. = WT-yeast + V. and Mnn9-yeast + V.; V. = *Vibrio campbellii*. Num.DF = numerator degrees of freedom; Den.DF = denominator degrees of freedom. For abbreviations, see Table 3.1.

Tests of fixed effects								
Strain	**GSL**				**VC**			
Effect	**Num. DF**	**Den. DF**	**F-value**	**P-value**	**Num. DF**	**Den. DF**	**F-value**	**P-value**
H/D Treatment	6	42	59.3	<.0001	6	42	34.4	<.0001
Type of feed + V.	1	42	55.8	<.0001	1	42	30.2	<.0001
H/D Treatment*Type of feed + V.	6	42	0.6	0.7700	6	42	0.3	0.9397
Time	1	154	2004.4	<.0001	1	154	1943.6	<.0001
Time*H/D Treatment	6	154	2.4	0.0331	6	154	6.8	<.0001
Time*Type of feed + V.	1	154	1.2	0.2778	1	154	0.03	0.8733
Time* H/D Treatment*Type of feed + V.	6	154	0.3	0.9357	6	154	0.2	0.9780



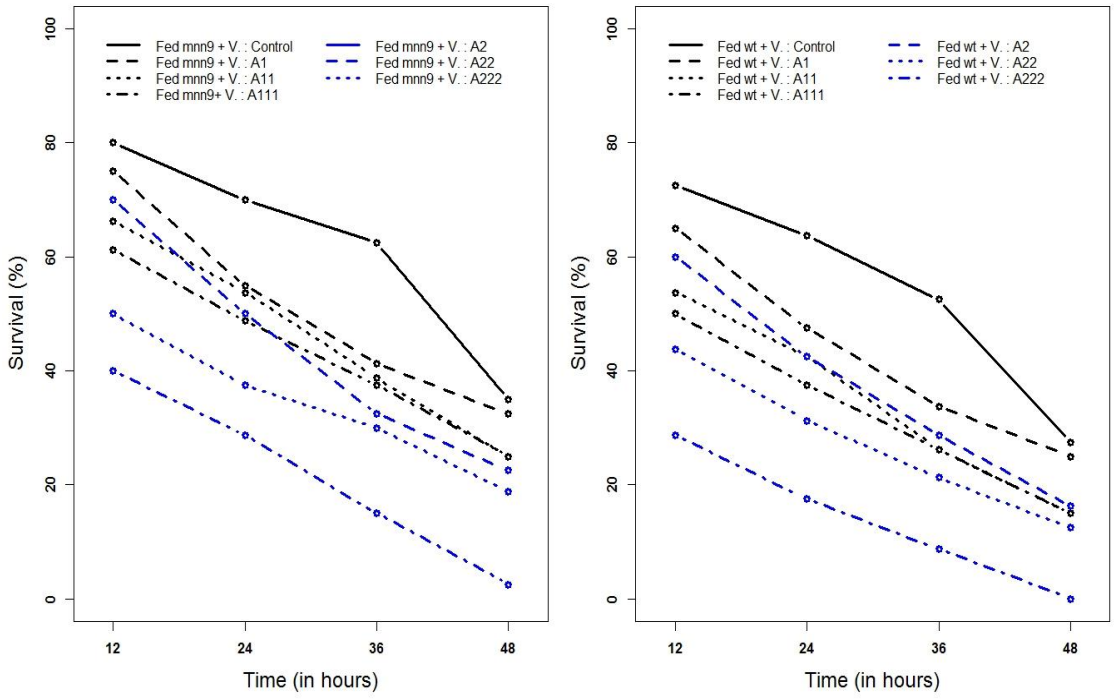


Figure 5.3: Line plot of survival (%) over a 48 h period of nauplii from **GSL** (top) and **VC** (below) cysts previously exposed to various H/D treatments (A1, A11, A111, A2, A22 and A222) at different time intervals (12, 24, 36 and 48 h). The nauplii were fed with yeast strains Wt (right), and Mnn9 (left) and supplemented with dead LVS3, and then challenged with *Vibrio campbellii* (+ V.). For abbreviations of H/D treatments, see Table 3.1.

5.4. Discussion

Most physiological and environmental stressors can impair the survival of cells and animals (Pedro et al., 1997; Braid et al., 2005; Varsamos et al., 2006). In addition, long-term stress increases susceptibility to infectious diseases, and this is also seen in fish (Peters et al., 1988) and shrimps (Lee and Wickins, 1992). Our aim was to determine if the negative effects of abiotic stress (produced by successive H/D treatments) on the survival and on the quality in general of *Artemia* nauplii, as shown in Chapter 3, can be remediated by selective nutrition by feeding the nauplii with two types of baker's yeast in combination with one bacterial strain. The beneficial effect of the tested diets on the growth and survival of *Artemia franciscana* nauplii was assessed under gnotobiotic conditions after their emergence from H/D-exposed cysts, as well as their resistance

to challenge with the pathogenic bacterium *Vibrio campbellii*. The utilization of gnotobiotic *Artemia* is important in this study in order to elucidate clearly the effects of the two baker's yeasts and the bacteria in the host, as any possible interference with the microbiota naturally present in the conventional culture system is eliminated in this model culture system, and therefore more conclusive results can be obtained.

Our results show that in general the yeast diet had a positive effect on the growth and survival of nauplii of the two *Artemia* strains tested after 2 days of culture. This result corresponds to earlier findings on the positive effects of a yeast cell diet on the survival and nutritional value of *Artemia* nauplii (Coutteau et al., 1990; Marques et al., 2004b). Moreover, the survival and growth of yeast (WT or Mnn9) fed *Artemia* nauplii emerging from the cysts exposed to a short period (2 h) of 1 or 2 cycles of hydration (*i.e.* A1 and A11 groups) or 1 cycle of 4 h of hydration (A2) was not significantly different from that of the control (emerging from untreated cysts). However, the two types of yeast feed could not improve the state of the nauplii and their survival was negatively affected when these nauplii emerged from cysts exposed to two or three H/D cycles of 4 h. This decrease in survival was most prominent in the case of fed nauplii, emerged from the A222 sample of the GSL strain, with 53 % decrease for the WT yeast diet and 50 % for the Mnn9 diet compared to the control after 2 days of culture. For the VC strain the decreases were 49 % and 48.4 % for WT and Mnn9 yeast, respectively. So the effect of the most extreme H/D treatments on the emerging *Artemia* nauplii could not be remediated by selective nutrition. Conversely, a short period of hydration/dehydration stress could be addressed through appropriate nutrition, using the two types of yeast in combination with bacteria.

In a subsequent experiment, the effect on survival of feeding two types of yeast to *Artemia* nauplii hatching from H/D-exposed cysts and challenged with pathogenic bacteria was investigated. The

results show that the feed composed of baker's yeast and bacteria provided protection against *Vibrio campbellii* during 48 h whether WT or Mnn9 was used, however with a significant difference between the two yeast strains fed. However, the survival after challenge was affected differentially by the different H/D treatments and by the incubation period: the pairwise interactions of the general mixed model demonstrated an interactive effect between on the one hand time and type of feed when *Vibrio* challenged, and on the other hand between H/D treatment and type of feed; the performance of the nauplii decreased in the order A1>A11>A111>A2>A22>A222 ($P < 0.05$). This indicates that the effect of the feed on the survival of the challenged nauplii depended on the H/D treatments and on the time. So the outcome of the challenge with *Vibrio campbellii* under gnotobiotic conditions was very much dependent on the overall condition of the nauplii, determined amongst others by the H/D treatment history of the cysts. Additionally, the GSL strain was more sensitive than VC to *V. campbellii*, and its performance and resistance to the pathogen was less enhanced by the food than in the VC strain. These differences could be related to genetic factors (Ruiz et al. 2007, 2008).

Our results showed that the non-challenged nauplii of both *Artemia* strains fed the Mnn9 mutant attained a statistically similar (not significantly different) survival compared to those fed WT yeast, whereas in case of challenge the Mnn9 strain resulted in significantly higher survival. So our results confirm previous studies that removal of the yeast cell wall by chemical treatment improves the nutritional value of yeast for *Artemia* (Coutteau et al., 1990). Marques et al. (2004b) showed that the Mnn9 yeast mutant, lacking a mannoprotein layer in the external cell wall, is more digestible by *Artemia* nauplii and always supported high biomass production and performance. Improved nitrogen assimilation in Mnn9 may also be related to the higher nutrient content of Mnn9, as indicated by its higher ash-free dry weight (Marques et al., 2004b; Soltanian et al., 2007).

In support of that, Toi (2014) reported that the use of Mnn9 as sole diet for *Artemia* nauplii results in remarkably higher nitrogen assimilation than when WT is used.

The methods by which bacteria are killed can influence their nutritional value. Autoclaving damages the cell wall by thermal denaturation of proteins essential for cell wall rigidity (Neyens and Baeyens, 2003), which weakens the wall (Klis et al., 2002) and thereby facilitates the digestion of the bacteria by *Artemia* enzymes. Both Marques et al. (2005) and our results show that feeding *Artemia* with autoclaved bacteria as a supplemented food can improve survival and performance of *Artemia*, especially when fed with poor-quality feeds such as WT (Marques et al., 2006c).

The literature indicates that in addition to being a dietary component themselves, bacteria can also improve the availability of yeast nutrients to *Artemia*. The provision of exogenous digestive enzymes by bacteria for *Artemia* has been reported previously (Intriago and Jones, 1993; Marques et al., 2004a). Bacterial enzymes, when released in the *Artemia* digestive tract, may improve yeast digestibility and improve its nutritional value for *Artemia* (Marques et al., 2006a) as well as for rotifers (Tinh et al., 2006). Incorporation of *Aeromonas hydrophila* LVS3 in the *Artemia* feed in our study may have provided such enzymes that facilitated the degradation of yeast and improved nutrient absorption by *Artemia*.

When starved *Artemia* nauplii, hatched from H/D-exposed cysts, were challenged with the relatively virulent *Vibrio campbellii*, the pathogen had a drastic effect on *Artemia* survival. For GSL, none of these starved nauplii survived after 48 h of culture, and the VC strain did not fare much better (0.0 % to 7.5 %). The overall mortality of the *Artemia* hatched from H/D-exposed cysts after 2 days of starvation is in agreement with the observations by Treece (2000) and Marques et al. (2005), which indicated that starved *Artemia* nauplii had consumed all yolk reserves and did

not survive in the absence of external food beyond the 4th day after hatching. This result also confirms our previous results (Chapter 3), where repeated H/D cycles resulted in reduced starved naupliar longevity and individual energy content, which could have reduced overall survival, fitness and resistance to infection.

In conclusion, our results provide the first evidence that a brief treatment with H/D cycles, unlike longer term treatments, does not have a deleterious effect on the resistance of emerged larvae to pathogenic *Vibrio* if they have been provided with an optimum diet. However, the effects on *Artemia* nauplii emerged from cysts exposed to a long period of H/D treatment are too severe to be addressed through the nutritional regime that we applied in our experiments. Moreover, the combination of yeast with a defective cell wall and bacteria as a feed for challenged *Artemia* nauplii after emergence from cysts subjected to H/D stress beneficially influenced the growth, survival and resistance towards a pathogen compared to the use of wild type yeast. The results suggest that the negative effects of abiotic stress on the survival and on the quality in general can be remediated by selective nutrition.

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Chapter 6

Effect of light colour, timing and duration of light exposure on the hatchability of *Artemia* cysts

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Abstract

We investigated the effect of illumination on the hatching of cysts of one strain of *Artemia franciscana* and two strains of parthenogenetic *Artemia* (Branchiopoda, Anostraca). The following light parameters were used: colour (red, blue and white light, having different intensities in the range 22 – 27 $\mu E \cdot m^{-2}\ s^{-1}$, corresponding with different wavelengths in the range 400 – 700 nm), and additionally (experiment 1) duration of light exposure (varying between 15 min and continuous light) or (experiment 2) timing of light exposure (from the 1st to the 13th hour of incubation of cysts for hatching). Continuous darkness was included as negative control. Hatching percentage was determined after 24 and 48 h, and additionally after 72 h in experiment 2. For all samples a relatively short exposure to light (6 h or less) during the initial hours of incubation maximally triggered the hatching process; 1 h of light had the highest efficiency when given during the 4th hour of incubation of eggs for hatching, and less so when supplied earlier or later. Lower sensitivity was observed for the red light spectral region (600 – 700 nm), with mostly limited differences between blue (400 – 500 nm) and white (400 – 700 nm) light. Differences between samples may be linked to factors such as chorion thickness, pigmentation, storage conditions, diapause status and genotypic differences in general, but to what extent each of these factors contributes to the variability among the strains needs to be studied by analyzing a more comprehensive set of samples. Nevertheless, the fact that the inter-strain differences observed in our study were only of quantitative nature suggests that light triggers hatching in *Artemia* cysts through a process that is consistent throughout the genus. Our work may contribute to a better understanding of the hatching biology of dormant life stages in Crustacea in general.

6.1. Introduction

The branchiopod crustacean *Artemia*, the brine shrimp, is the most widely used live food organism in larviculture of fish and shellfish. The importance of *Artemia* as live food lies in its flexibility in use and in the ease with which its resting eggs can be stored and transported without loss of viability (Lavens et al., 1986). Although most *Artemia* cysts available on the world market are harvested from the Great Salt Lake (USA), several resources, being either bisexual or parthenogenetic populations, are exploited worldwide (Lavens and Sorgeloos, 2000). Hatching output of the cysts is an important quality criterion but may be very variable. Such variations arise from differences in harvesting, processing and storage techniques (Vos et al., 1984) and are also partially related to the phenomenon of metabolic arrest known as diapause. The state of diapause may be terminated by several environmental cues (*e.g.* desiccation, hibernation), after which the embryos are in a state called quiescence and wait to resume development and to hatch under appropriate environmental conditions (*e.g.* in terms of hydration level, temperature, oxygen) (Drinkwater and Crowe, 1987; Robbins et al., 2010).

Light is an important factor affecting various life processes in *Artemia* and in crustaceans in general. The mode of *Artemia* reproduction is influenced by environmental factors such as ambient salinity and oxygen levels (Clegg and Trotman, 2002), but also by the photoperiod in combination with temperature (Nambu et al., 2004): short days and higher temperature favour oviparity (production of resting eggs), whereas long days and lower temperature promote ovoviviparity (release of free-swimming nauplii). The length and sequence of light and dark phases is also an important determinant of hatching in other crustaceans, such as in cladoceran eggs (Vandekerkhove et al., 2005). As in other branchiopod crustacean eggs (Pancella and Stross, 1963; Bishop, 1967; Hempel-Zawitkowska, 1970; Takahashi, 1975; Mitchell, 1990; Murugan and

Dumont; 1995; Horiguchi et al., 2009), light is needed to induce hatching in *Artemia* embryos (Sorgeloos, 1973). Several studies tried to unravel the physiological processes triggered by light exposure (Van Der Linden et al., 1985; 1986; 1987; 1988; 1991). According to Vanhaecke et al. (1981), hatching increases with light intensity over a range of 20 to 2000 lux of permanent illumination, but remains constant beyond that point. Branchiopod embryos are activated by a broad spectrum of wavelengths, from ultraviolet (395 nm) to red (660 nm) (Kashiyama et al., 2010), which suggests the involvement of different optical pigments in photoreception. Darkness also promoted embryonic diapause termination of dormant *Artemia* eggs (Nambu et al., 2008; 2009).

In general, experiments with *Artemia* have been performed so far with commercial *Artemia franciscana* Kellogg 1906 samples. Generally one aspect, such as light colour or intensity was analyzed at a time. Our objective was to study how light triggers the hatching process by introducing treatments with different colour, timing and duration of light exposure. We wanted to test the hypothesis that there are specific periods during the incubation for hatching, in which the *Artemia* embryo is more susceptible to the light trigger. We also assumed that the effect would be different when using light of different colour (*i.e.* wavelength and energy). We finally wanted to investigate if the effects would be different when using different samples belonging to different strains. Through this study we aimed to contribute to the overall understanding of the hatching physiology in the genus *Artemia*, and in life cycle stages of Crustacea in general.

6.2. Materials and methods

6.2.1. Cyst samples

Experiments were performed with three samples of dried encysted *Artemia* cysts: the first sample belonged to the bisexual species *Artemia franciscana* (Kellogg, 1906) and was the strain originating from Vinh Chau (VC) salt fields, Vietnam (ARC code 1742), collected in 2009. A second sample was parthenogenetic *Artemia* (Barigozzi, 1974; Bowen and Sterling, 1978) from Tuz Lake (TK), Kazakhstan (ARC code 1761, collected in 2005), and the third sample was parthenogenetic *Artemia* from Bolshoye Yarovoye Lake (BY), Siberia, Russia (ARC code 1758, collected in 2005). All samples had been obtained through local contact persons, and had been stored at + 4 °C in darkness since their arrival at the Laboratory of Aquaculture & *Artemia* Reference Center (ARC), Ghent University, Belgium. Samples of these strains were available to us in sufficient quantities to run the tests. They showed very different hatching in standard hatching conditions (*i.e.* continuous white light; Van Stappen, 1996) in a first screening test over 24 h (*i.e.* 90.2, 57.4 and 33.2 % after 24 h for VC, BY and TK, respectively), which made them an interesting set to compare under varying light conditions. Finally VC and BY are also commercially available strains, so they were also chosen because we expected our findings to have implications for routine hatching operations.

6.2.2. Experimental design

All hatching experiments were done in triplicates and hatching conditions (except for light) were kept optimal (Van Stappen, 1996). Cysts (0.05 g) were incubated for hatching in sterile 50 mL screw-cap falcon tubes containing 25 mL of Instant Ocean® solution of 32 g L^{-1} salinity. They

were kept in suspension on a rotor at 4 cycles per min to prevent clogging and sedimentation of the eggs in a room at 28 ± 1 °C.

Fluorescent lamps (Philips TL-D 18W, SLV) generating red (600 – 700 nm), blue (400 – 500 nm) or white (400 – 700 nm) light within the visible spectrum, according to the manufacturer's specifications, were positioned 20 cm above the hatching set-up. The different light treatments were shaded from each other. The light intensity (expressed as $\mu E \cdot m^{-2}\ s^{-1}$) reaching the surface of the hatching medium was measured with a light meter (LI-Cor sensor-190) and was 27, 24 and 22 $\mu E \cdot m^{-2}\ s^{-1}$ for red, blue and white light, respectively.

In a first experiment, we tried to determine the minimum duration of light exposure, needed to obtain maximal hatching for the three strains and the three light colours. For this purpose, over an incubation period of 48 h different durations of light exposure were tested (Table 6.1): 15 min, 30 min, 1.0 h, 1.5 h, 2.0 h, 4.0 h and 6.0 h of light from the start of incubation onwards. The period of light exposure was followed by darkness until the completion of the 48 h. Continuous light over 48 h was used as a positive control and continuous darkness for 48 h as a negative control. Hatching was recorded after 24 and 48 h.

Table 6.1: Overview of experimental setup: dark cells correspond to exposure to darkness, white cells to light exposure. Hatching was evaluated after 24 and 48 h total incubation for hatching in experiment 1, and after 24, 48 and 72 h incubation for hatching in experiment 2 (n/a = not applied). Width of three rightmost columns is not proportional to actual duration.

Time (h) over 72 h incubation period for hatching	0-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-24	24-48	48-72
Experiment 1: Different duration of light exposure (from 15 min to 6 h)																
positive control																n/a
negative control																n/a
15 min light																n/a
30 min light																n/a
1.0 h light																n/a
1.5 h light																n/a
2.0 h light																n/a
4.0 h light																n/a
6.0 h light																n/a
Experiment 2: Different timing of 1 h light exposure																
positive control																
negative control																
1st h light																
4th h light																
8th h light																
13th h light																

In a second experiment we tried to identify in which period of the incubation process light exposure proved most efficient in inducing hatching. Therefore, for each colour a relatively short exposure of 1 h of light was given: during the 1st hour (1 h starting from the onset of incubation for hatching onwards, during which the cysts achieve full hydration), 4th, 8th or 13th hour of the incubation period (Table 6.1). The total incubation period for hatching in experiment 2 was prolonged up to 72 h, because the first experiment had shown some limited hatching increase from 24 h to 48 h. We thus assumed that 48 h might not be enough for a complete assessment of the hatching process. Eggs were kept in darkness during the remaining of the 72 h incubation period. Continuous darkness and continuous light over 72 h were included as negative and positive controls, respectively. Hatching was recorded after 24, 48 and 72 h.

6.2.3. Determination of hatching percentage (H %)

Hatching percentage was determined according to standard procedures (Van Stappen, 1996). After 24 h and 48 h of incubation (and additionally after 72 h in the second experiment), six subsamples of 250 µL each were taken from each falcon tube with a micropipette and placed in a small vial. Nauplii were fixed by adding a few drops of lugol solution and tap water. The nauplii as well as the umbrellae (emerged larvae still surrounded by the hatching membrane) were counted under the microscope. The unhatched cysts were subsequently decapsulated by adding a few drops of NaOCl and NaOH solution to each vial (Bruggeman et al., 1980), and the orange colored non-hatched embryos were counted.

The hatching percentage was calculated as follows (modified from Van Stappen, 1996: the embryos in the umbrella stage were considered as having reacted to the light trigger, and were thus included in the calculation of the hatching percentage):

H % = N + U/ (N + U + E) x 100, where N = number of nauplii, U = number of umbrellae, E = number of embryos.

The mean hatching value per falcon tube was recorded and the overall mean hatching percentage and standard error for the three replicate falcon tubes were calculated.

As different strains may have different chorion thickness and as this may interfere with light sensitivity, the chorion thickness was determined. Therefore, the diameter of non-decapsulated and decapsulated eggs was measured according to Abatzopoulos *et al.* (2006). For this purpose, 1 g of cysts was fully hydrated by incubation for 2 h in freshwater and then fixed in 1 % lugol's solution overnight. The diameter of minimum 100 cysts was measured using a light microscope equipped with a calibrated eyepiece. Analogously the diameter of minimum 100 decapsulated cysts (decapsulated according to Van Stappen, 1996, followed by overnight fixation with lugol) was determined. Average values of non-decapsulated and decapsulated cysts were calculated and then chorion thickness was calculated as follows:

Chorion thickness = (mean diameter of non-decapsulated - mean diameter of decapsulated cysts)/2.

6.2.4. Statistical analysis

A hierarchical generalized linear mixed model (HGLMM) with a binomial distribution and a logit link was fitted to the hatching data. Strain, light colour and incubation time were set as fixed effects in fitting the HGLMM to the control data (continuous darkness and continuous light). For each incubation period separately, strain, light colour, duration and timing of light exposure were set as fixed effects in fitting the HGLMM to the duration and timing data, respectively. In all HGLMMs fitted, "hatching recipient" was set as additional random blocking factor with a beta distribution

and logit link. The fixed effects as well as the variance components associated with the random effects were estimated using the residual maximum likelihood (REML) as implemented in Genstat v17 (Payne 2014). Significance of the fixed main and interaction effects was assessed by an F-test. Post-hoc pairwise comparisons of 24 h values were tested for significance based on least significant differences (LSD) at the 5 % level.

6.3. Results

Chorion thickness was lower in cysts from VC (6.4 µm) than in those from BY (11.4 µm) and TK (12.2 µm).

In experiment 1, the hatching percentage of cysts exposed to 24 h continuous white light was highest for VC (93.1 %), moderate for BY (56.1 %) and lowest for TK (36. 1%) (Fig. 6.1. A, 6.2. A and 6.3. A, respectively). The 24 h values were lower when using continuous blue light (85.4, 48.8 and 19.1 % for VC, BY and TK, respectively) than with white light, and when using red light they were the lowest (49.6, 19.9 and 10.7 %, respectively). In darkness, hatching was lower than under any light source (21.2, 7.9 and 3.7, respectively, after 24 h) (Fig. 6.1 – 6.3 A).

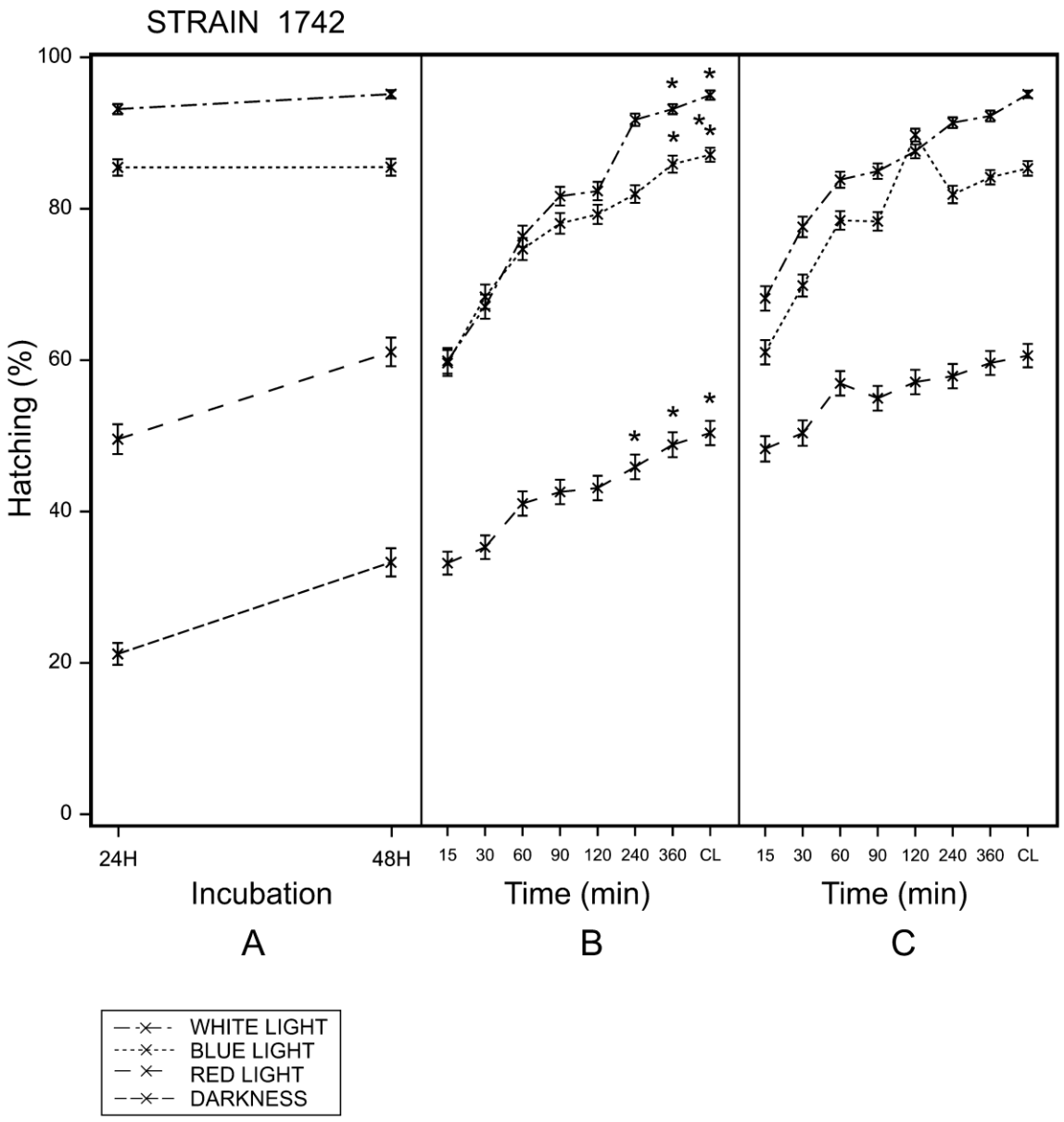

Figure 6.1: Experiment 1. Plots of mean percentage of hatching ± standard error, measured in **strain 1742 (VC)**: per incubation period (24 h and 48 h) under continuous white, blue and red light or darkness (**A**); for different duration of light exposure using white, blue and red light, incubated for 24 h (**B**) and 48 h (**C**). CL = continuous light. For each colour, asterisks (*) above points in graph B indicate values not significantly different (LCD post-hoc, $P < 0.05$) from the corresponding CL value.

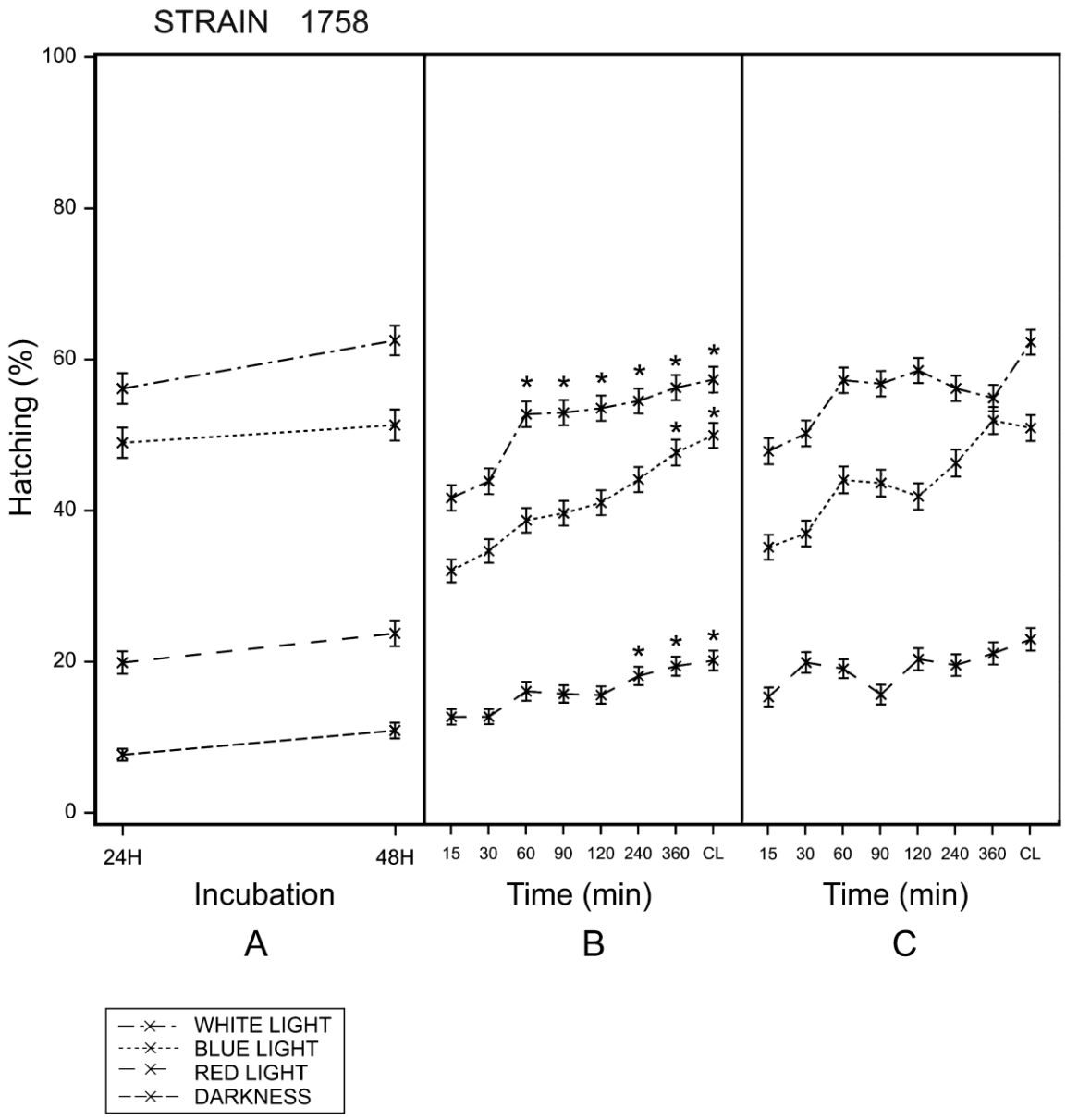

Figure 6.2: Experiment 1. Plots of mean percentage of hatching ± standard error, measured in **strain 1758 (BY)**: per incubation period (24 h and 48 h) under continuous white, blue and red light or darkness (**A**); for different duration of light exposure using white, blue and red light, incubated for 24 h (**B**) and 48 h (**C**). CL = continuous light. For each colour, asterisks (*) above points in graph B indicate values not significantly different (LCD post-hoc, $P < 0.05$) from the corresponding CL value.

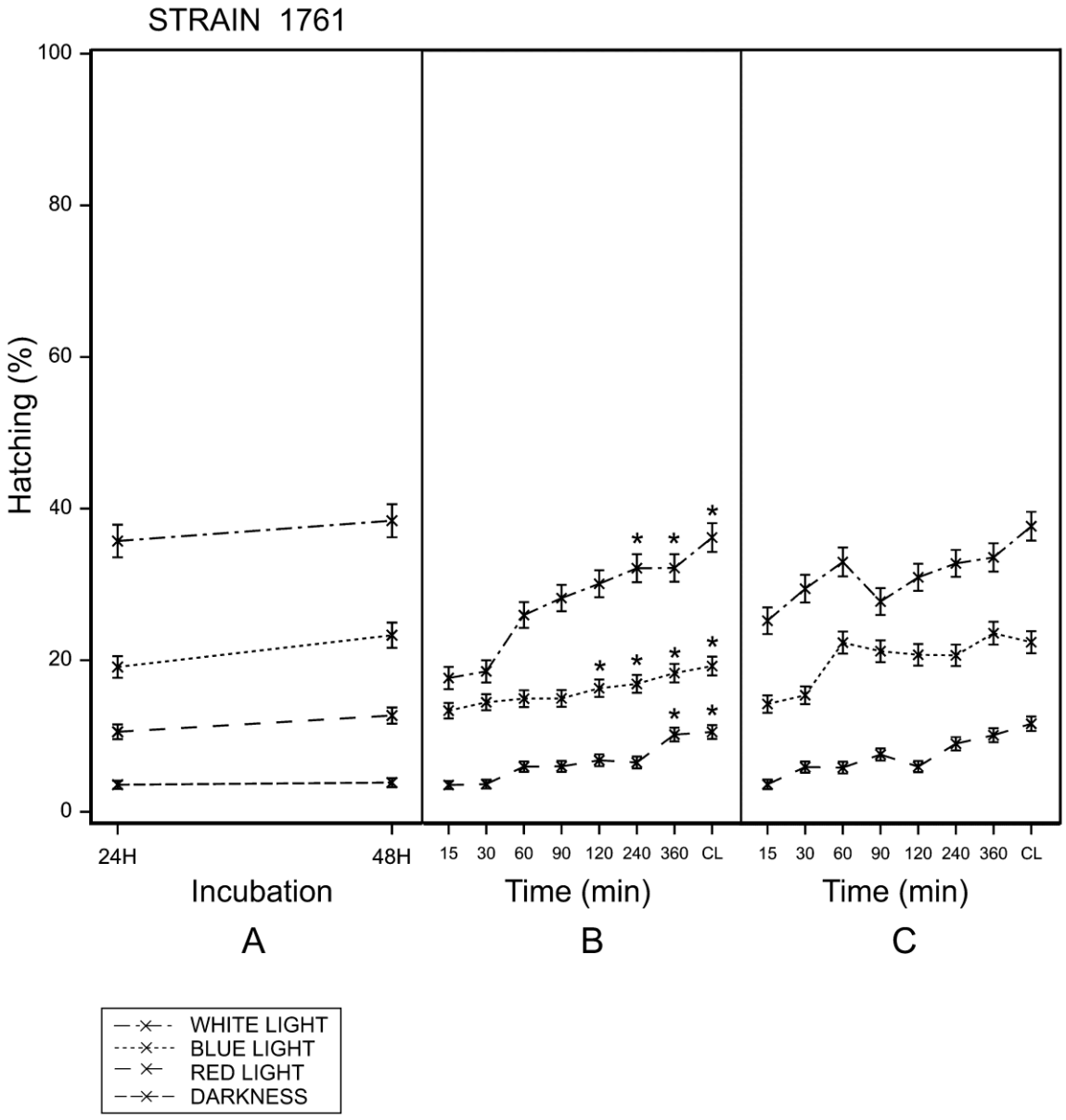

Figure 6.3: Experiment 1. Plots of mean percentage of hatching ± standard error, measured in **strain 1761 (TK)**: per incubation period (24 h and 48 h) under continuous white, blue and red light or darkness (**A**); for different duration of light exposure using white, blue and red light, incubated for 24 h (**B**) and 48 h (**C**). CL = continuous light. For each colour, asterisks (*) above points in graph B indicate values not significantly different (LCD post-hoc, $P < 0.05$) from the corresponding CL value.

HGLMM of the control data (continuous light of different colours and continuous darkness) showed that the period of incubation of cysts for hatching (24 or 48 h), as well as strain and light colour, had a significant effect ($P < 0.05$) on the hatching values (Table 6.2 a). Moreover, there was a significant interaction ($P < 0.05$) between strain and light colour. Other interactions were

not significant ($P > 0.05$) (Table 6.2 a). All pairwise comparisons (LCD test; within each strain) between 24 h continuous light values, obtained with different colours in experiment 1, showed significant difference ($P < 0.05$).

Table 6.2: Experiment 1: Statistics of tests for fixed effects (hierarchical generalized linear mixed model) (n.d.f. = numerator degrees of freedom; d.d.f. = denominator degrees of freedom; pr = probability).

(a) Effects of hatching incubation time, strain, light colour, and their interactions, on hatching values in controls (continuous light and darkness)

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Incubation time	26.9	1	26.9	41.0	<0.001
Strain	2111.1	2	1055.4	45.5	<0.001
Light colour	3248.5	3	1082.6	44.2	<0.001
Incubation time x strain	3.6	2	1.8	45.7	0.173
Incubation time x light colour	8.9	3	3.0	44.3	0.042
Strain x light colour	130.2	6	21.7	51.3	<0.001
Incubation time x strain x light colour	8.5	6	1.4	51.4	0.228

(b) Effects of strain, light colour and duration of light exposure and their interactions, on hatching values after 24 h in treatments with different duration of light exposure

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Strain	8946.6	2	4472.6	144.4	<0.001
Light colour	5659.9	2	2829.9	129.8	<0.001
Duration of light exposure	1165.2	7	166.5	121.6	<0.001
Strain x light colour	92.8	4	23.2	157.1	<0.001
Strain x duration	144.3	14	10.3	147.3	<0.001
Light colour x duration	95.1	14	6.8	131.3	<0.001
Strain x light colour x duration	171.3	28	6.1	160.3	<0.001

(c) Effects of strain, light colour and duration of light exposure and their interactions, on hatching values after 48 h in treatments with different duration of light exposure

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Strain	10510.1	2	5254.6	146.7	<0.001
Light colour	4783.0	2	2391.5	136.9	<0.001
Duration of light exposure	756.4	7	108.1	128.9	<0.001
Strain x light colour	6.6	4	1.7	156.5	0.165
Strain x duration	127.2	14	10.0	149.6	<0.001
Light colour x duration	88.5	14	6.3	138.9	<0.001
Strain x light colour x duration	210.4	28	7.5	159.5	<0.001

HGLMM analysis of the 24 h hatching values, obtained with different duration of light exposure in experiment 1, showed a significant effect ($P < 0.05$) of strain, light colour and duration of light exposure on hatching (Table 6.2 b). Also all interactions between all factors were significant. This was also the case for the 48 h hatching values (Table 6.2 c), except for the interaction between

light colour and strain, which was not significant after 48 h ($P > 0.05$). The results obtained under white light were generally higher than the corresponding values obtained under blue light; this was the case for any duration of light exposure, any strain and both for 24 h (Fig. 6.1 – 6.3 B) and 48 h (Fig. 6.1 – 6.3 C) values. Red light values were the lowest. All strains and colours showed a gradual increase in 24 h hatching values as the duration of light exposure increased from 15 min to 24 h continuous light. After 15 min of exposure to white light, the 24 h hatching values were 58.4 % for VC (Fig. 6.1 B), 41.0 % for BY (Fig. 6.2 B) and 17.6 % for TK (Fig. 6.3 B). If light exposure was prolonged to 6 h, the respective values were 91.3 %, 55.3 % and 32.2 %. Further prolongation from 6 h to 24 h resulted in minor (less than 5 %) increase in H %. The values obtained after 48 h showed a similar pattern, although with a less steady increase of hatching as light exposure was prolonged (Fig. 6.1 – 6.3 C).

Through LCD post-hoc pairwise comparison, we calculated the minimal duration of light exposure in experiment 1, needed to obtain a 24 h hatching value similar to that obtained under continuous light. This minimum duration was different, depending on light colour and strain. A minimum of 4 h of red light was needed for VC (Fig. 6.1 B) and BY (Fig. 6.2 B) and even 6 h for TK (Fig. 6.3 B). Minimum 6 h of blue light was needed for BY and VC, whereas 2 h of blue light was sufficient for TK. For white light, finally, the minimum duration was 1, 4 and 6 h for BY, TK and VC, respectively.

In experiment 2, the values obtained with the controls (continuous light or darkness for 24 and 48 h; results not shown) were similar to those in experiment 1; prolongation of the incubation period from 48 h to 72 h resulted in less than 1 % of hatching increase, if any. In experiment 2, strain, light colour and incubation time all had a significant effect (HGLMM, $P < 0.05$) on the control values. The interactions incubation time-light colour and strain-light colour were significant as

well ($P < 0.05$) (Table 6.3 a). Also in experiment 2 all pairwise comparisons (LCD test; within each strain) of 24 h continuous light values, obtained with different light colours, showed significant difference ($P < 0.05$).

Table 6.3: Experiment 2: Statistics of tests for fixed effects (hierarchical generalized linear mixed model) (n.d.f. = numerator degrees of freedom; d.d.f. = denominator degrees of freedom; pr = probability).

(a) Effects of hatching incubation time, strain, light colour, and their interactions, on hatching values in controls (continuous light and darkness)

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Incubation time	26.1	2	13.1	63.4	<0.001
Strain	2033.4	2	1016.5	74.6	<0.001
Light colour	8852.5	3	2950.3	69.0	<0.001
Incubation time x strain	17.3	4	4.3	75.3	0.003
Incubation time x light colour	28.4	6	4.7	69.5	<0.001
Strain x light colour	2011.6	6	334.9	83.4	<0.001
Incubation time x strain x light colour	12.3	12	1.0	84.2	0.435

(b) Effects of strain, light colour and timing of light exposure and their interactions, on hatching values after 24 h in treatments with different timing of light exposure

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Strain	2548.9	2	1274.3	87.7	<0.001
Light colour	6945.8	2	3472.7	83.0	<0.001
Timing of light exposure	656.6	4	164.1	77.1	<0.001
Strain x light colour	2553.1	4	637.9	97.6	<0.001
Strain x timing	70.2	8	8.8	89.3	<0.001
Light colour x timing	104.9	8	13.1	84.0	<0.001
Strain x light colour x timing	136.2	16	8.5	99.2	<0.001

(c) Effects of strain, light colour and timing of light exposure and their interactions, on hatching values after 48 h in treatments with different timing of light exposure

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Strain	3787.6	2	1893.5	100.1	<0.001
Light colour	7612.0	2	3805.8	90.6	<0.001
Timing of light exposure	419.1	4	104.8	82.9	<0.001
Strain x light colour	2956.5	4	738.7	110.8	<0.001
Strain x timing	60.1	8	7.5	101.7	<0.001
Light colour x timing	71.0	8	8.8	91.7	<0.001
Strain x light colour x timing	106.9	16	6.7	111.8	<0.001

(d) Effects of strain, light colour and timing of light exposure and their interactions, on hatching values after 72 h in treatments with different timing of light exposure

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Strain	3442.17	2	1720.9	94.1	<0.001
Light colour	6420.3	2	3210.1	88.4	<0.001
Timing of light exposure	275.7	4	68.9	83.1	<0.001
Strain x light colour	2448.7	4	612.0	100.5	<0.001
Strain x timing	62.2	8	7.8	95.4	<0.001
Light colour x timing	42.7	8	5.3	89.3	<0.001
Strain x light colour x timing	92.5	16	5.8	101.4	<0.001

HGLMM analysis of the 24 h values, obtained in experiment 2 with different timing of light exposure, showed a significant effect ($P < 0.05$) of strain, light colour and timing of light exposure on hatching (Table 6.3 b). In addition, all interactions between all factors were significant. This was also the case for the 48 h and 72 h values (Table 6.3 c and 6.3 d, respectively). Also in experiment 2 the hatching in any strain after 24 h (Fig. 6.4 – 6.6 A), 48 h (Fig. 6.4 – 6.6 B), and 72 h (Fig. 6.4 – 6.6 C) and with any timing of light exposure was always higher under white light than under blue light, whereas red light values were the lowest. If white light exposure was delayed from the 1st to the 4th hour, the hatching percentage after 24 h of incubation increased from 68.9, 48.3 and 17.7 % (for VC, BY and TK, respectively) up to 84.2 % (Fig. 6.4 A), 53.5 % (Fig. 6.5 A) and 24.1 % (Fig. 6.6 A), respectively. Further delay of white light exposure to the 8th hour, however, resulted in a reduction of hatching as compared with the 4th hour to levels similar to the 1st hour values. Postponing white light exposure to the 13th hour further reduced hatching. This trend was also observed for blue and red light in VC and BY. In TK, however, postponing blue or red light exposure over the first 13 h of the incubation for hatching resulted in a slight but gradual increase of hatching (Fig. 6.4 – 6.6 A). In general, prolongation of incubation to 48 h (Fig. 6.4 – 6.6 B) or 72 h (Fig. 6.4 – 6.6 C) generally resulted in minor hatching increase, if any, as compared to 24 h values.

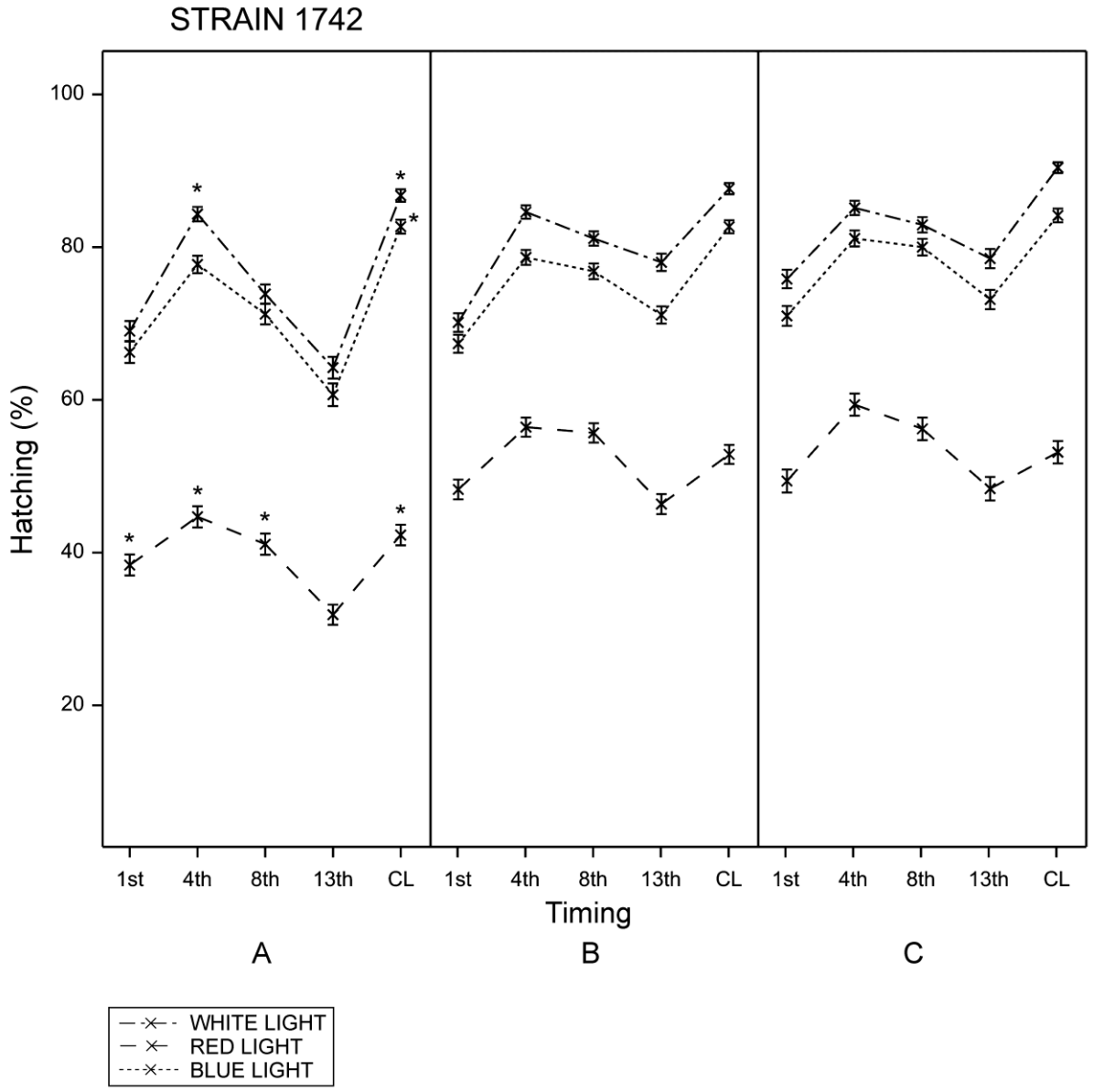

Figure 6.4: Experiment 2. Plot of mean percentage of hatching ± standard error, measured in **strain 1742 (VC)** for different timing of light exposure (x^{th} hour after onset of hatching incubation) using white, red and blue light, incubated for 24 h (**A**), 48 h (**B**), 72 h (**C**). CL = continuous light. For each colour, asterisks (*) above points in graph A indicate values not significantly different (LCD post-hoc, $P < 0.05$) from the corresponding CL value.

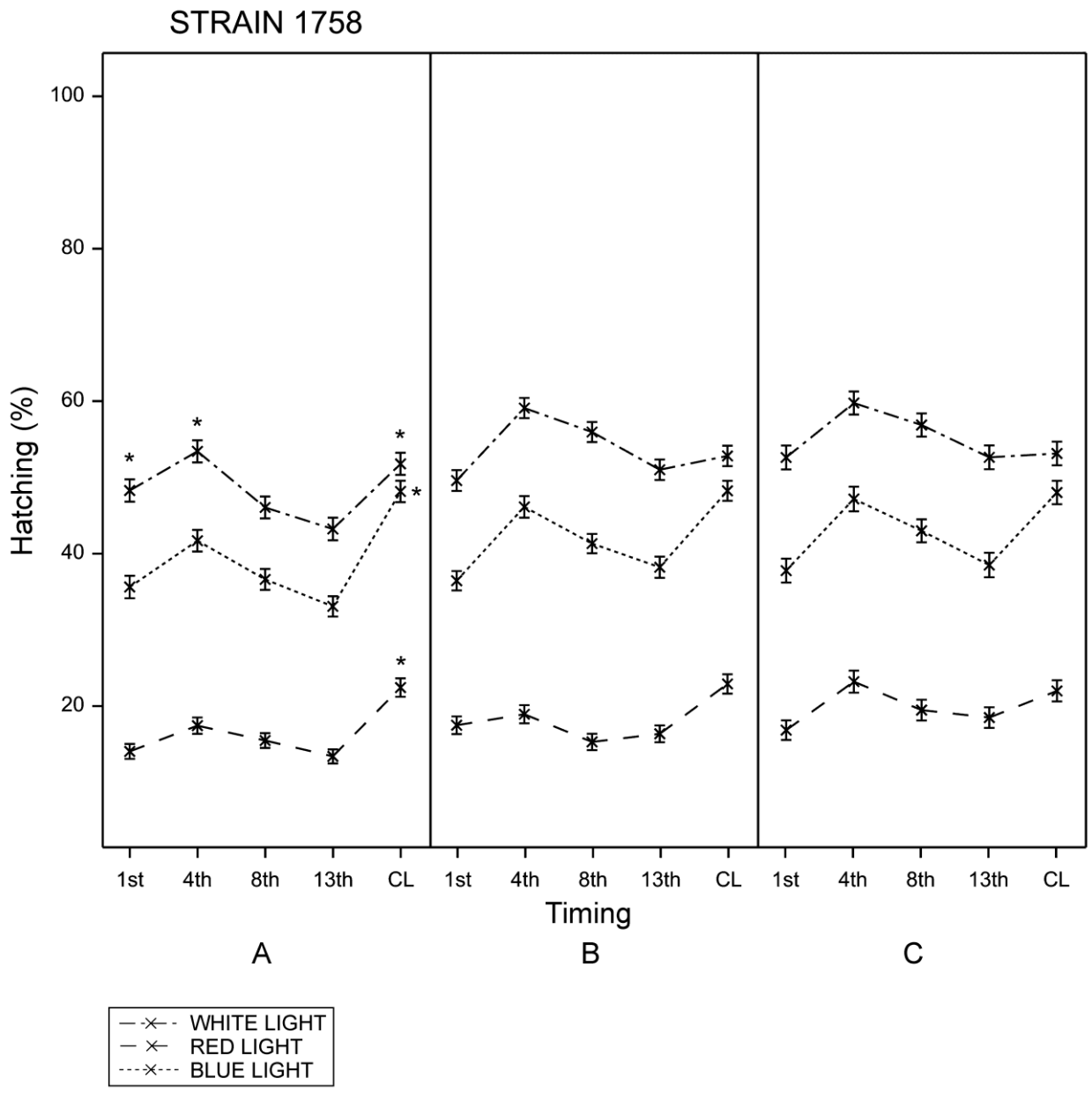

Figure 6.5: Experiment 2. Plot of mean percentage of hatching ± standard error measured in **strain 1758 (BY)** for different timing of light exposure (x^{th} hour after onset of hatching incubation) using white, red and blue light, incubated for 24 h (**A**), 48 h (**B**), 72 h (**C**). CL = continuous light. For each colour, asterisks (*) above points in graph A indicate values not significantly different (LCD post-hoc, $P < 0.05$) from the corresponding CL value.

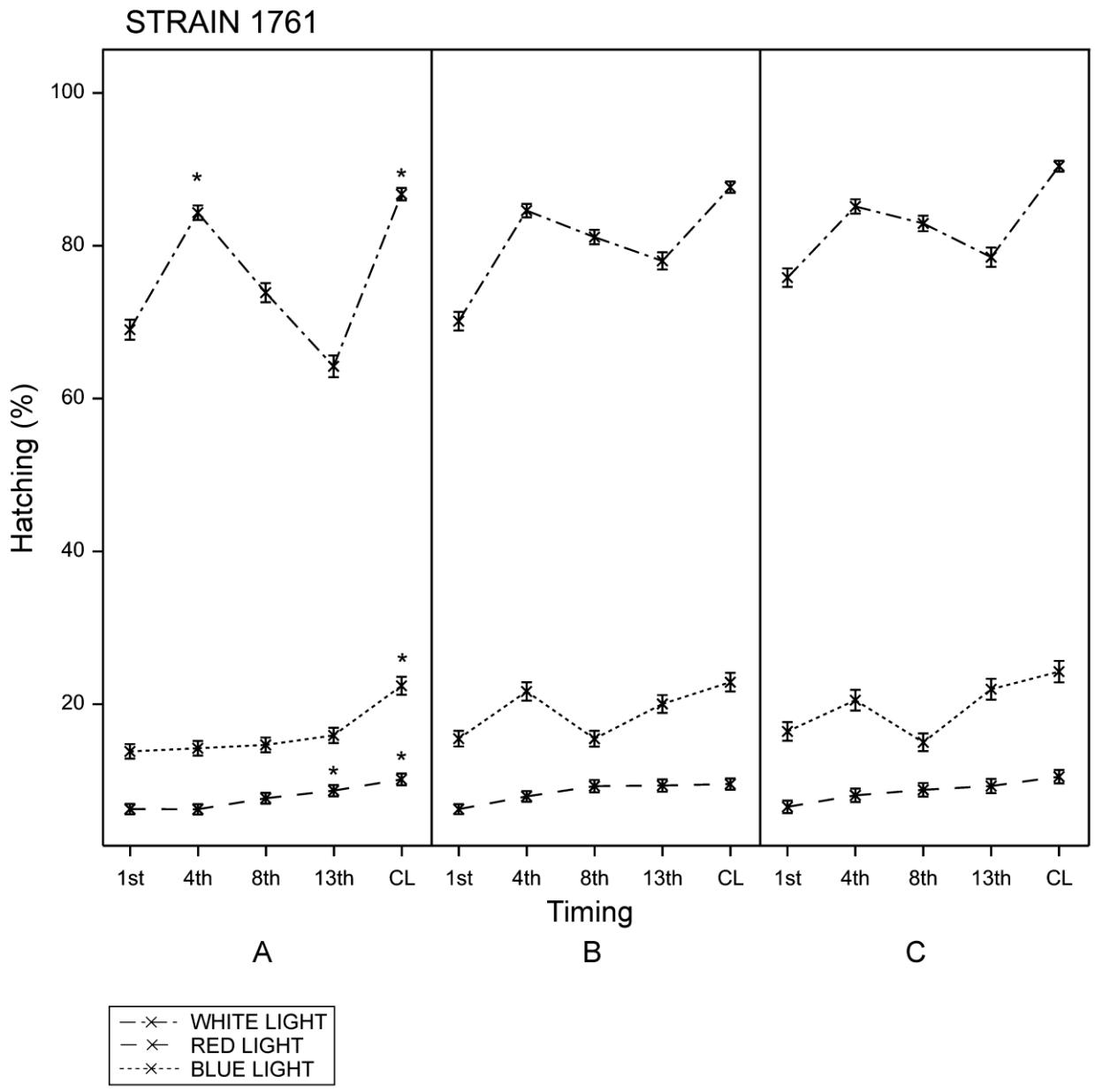

Figure 6.6: Experiment 2. Plot of mean percentage of hatching ± standard error measured in **strain 1761 (TK)** for different timing of light exposure (x^{th} hour after onset of hatching incubation) using white, red and blue light, incubated for 24 h (**A**), 48 h (**B**), 72 h (**C**). CL = continuous light. For each colour, asterisks (*) above points in graph A indicate values not significantly different (LCD post-hoc, $P < 0.05$) from the corresponding CL value.

Through LCD post-hoc pairwise comparison, we calculated if 1 h of light exposure, depending on its timing, resulted in 24 h hatching values similar to those obtained under continuous light. As in experiment 1, the results were different depending on the strain and the light colour used. One hour

of red light was maximally efficient (not significantly different from the positive control) when supplied during the 13th hour for TK (Fig. 6.6 A), the 1st, 4th or 8th hour for VC (Fig. 6.4 A), and was never as efficient as continuous light in BY (Fig. 6.5 A). One hour of blue light never resulted in hatching as high as continuous blue light. One hour of white light, finally, resulted in all strains (Fig. 6.4 – 6.6 A) in hatching similar to the control when supplied during the 4th hour and additionally during the 1st hour for BY (Fig. 6.5 A).

6.4. Discussion

Light-induced hatching has been widely reported in branchiopod crustaceans, with different light conditions (*e.g.* in terms of light intensity, photoperiod) resulting in different hatching success (Pancella and Stross, 1963; Bishop, 1967; Hempel-Zawitkowska, 1970; Shan, 1970; Sorgeloos, 1973; Takahashi, 1977; Vanhaecke et al., 1981; Mitchell, 1990; Murugan and Dumont, 1995). Despite the commercial interest of aquaculture farms in high hatching outputs of brine shrimp cysts and thus the need to have maximal understanding of the hatching metabolism, only limited efforts have been made to unravel the exact role of light during the hatching process in *Artemia* (Sorgeloos, 1973; Royan, 1976; Vanhaecke et al., 1981; Van der Linden et al., 1985; 1986).

Our aim was to determine the minimum duration of light exposure needed to achieve maximal hatching, and to assess if there is different sensitivity to light at different phases of the incubation process for hatching. We investigated this for three light colours with different intensities in the range 22 ~ 27 $\mu E \cdot m^{-2} s^{-1}$, corresponding with different wavelengths between 400 and 700 nm and using three strains of *Artemia*.

We found that in general white or blue light resulted in higher hatching output than red light, and in general a light exposure of 6 h (or even less, depending on the strain and the light colour) from

the start of incubation onwards resulted in maximal hatching. Extension of light exposure over a continuous 24 or 48 h period resulted in only limited increase in hatching.

In a next experiment we investigated at which stage of the incubation for hatching a relatively short exposure of 1 h has maximal effect. The observation period was prolonged to 72 h. We found that 1 h of white light was most effective when supplied during the 4th hour of incubation (hence well beyond complete hydration) and suboptimal when supplied earlier, *i.e.* during the 1st hour, while hydration of the cysts takes place or later (during the 8th hour, and especially the 13th hour). Results with blue and red light were less conclusive, but in general 1 h of blue or red light, provided during the 4th hour of hatching incubation resulted in higher hatching than 1 h of light provided earlier or later. Dry encysted *Artemia* cysts (such as in our samples) reach full hydration within about 2 h incubation in seawater of 32 g L^{-1} (Lavens and Sorgeloos, 1987). Sorgeloos (1973) found that the hatching trigger is related to the fully hydrated state of the cysts. Light is hence maximally effective after complete hydration of the cyst (as also shown for freshwater fairy shrimp by Pinceel et al., 2013). This period corresponds with the early hours of the onset of metabolism (Morris, 1971; Lavens and Sorgeloos, 1987). Within the context of a fish or shellfish hatchery, where hatching of *Artemia* cysts is a daily routine, combining the results of experiment 1 and 2 thus shows that, after complete hydration, cysts are more sensitive to illumination and that light should thus be provided after full hydration has been reached (optimally 3 to 5 hours after the start of incubation of dry cysts for hatching, which corresponds to more or less 1 to 3 hours after full hydration). By limiting our observation period to 48 h and 72 h (experiment 1 and 2, respectively) we have no insight in the numbers of embryos hatching extremely slowly, after this period. But as the hatching increase was limited when the incubation period was prolonged from 24 h to 48 h, and even less (if any) when prolonged to 72 h, we assume that the hatching values beyond 72 h

will not change our major conclusions. Moreover, from an aquaculture perspective, only the hatching observed during the first 24 h of the incubation for hatching is generally of practical relevance.

As in other branchiopod crustacean eggs (Brendonck and De Meester, 2003) only a fraction of *Artemia* embryos responded to darkness, except for considerable hatching (up to 30 % after 48 h) obtained with the VC strain in these conditions. Sensitivity to hatching cues may vary with strain or genotype as noted for the resting eggs of some monogonont rotifers and copepods (Pancella and Stross, 1963). Variations between strains may be attributed to differences in shell characteristics as well. Access of most molecules (*i.e.* water and gases) to *Artemia* embryos is restricted by the shell, a multi-layered chitinous structure (Anderson et al., 1970; Morris and Afzelius, 1967; Clegg, 1986; Clegg et al., 1996). The shell of crustacean zooplankton resting eggs contains the light-absorbing haemopigment, haematin (Van der Linden et al., 1986; 1988). The three strains used in our experiments showed high variation in chorion thickness, with the VC cysts having a chorion about half the thickness of the other two strains, but it is not clear if this is linked to the relatively high hatching of the VC strain in darkness. Moreover, photosensitivity may also be related to pigment intensities in the shell, which were not quantified in our study. The degree of shell pigmentation has been shown to affect the timing of hatching in the freshwater fairy shrimp *Branchipodopsis wolfi* (Pinceel et al., 2013). In *Artemia* prolonged exposure to sunlight in humid circumstances causes oxidation or bleaching of the haematin pigments in the shells, allowing for further light penetration over time (Van der Linden et al., 1986).

The VC sample was also different from the other two in being more recently harvested (2009 for VC versus 2005 for the others). Storage of zooplankton eggs in general may affect their hatching characteristics (Brendonck and De Meester, 2003) and thus maybe also their sensitivity for a light

trigger. All samples had been stored in identical conditions (in dark at + 4 °C) since their arrival in our laboratory, but this storage period was much shorter in case of VC. Moreover, the pre-shipment storage conditions were out of our control and most probably had not been the same for the different samples. It cannot be excluded that these antecedents have an effect on light sensitivity. Finally, the different strains used in our study may have been in a different state of diapause, as suggested by the different hatching observed in standard hatching conditions (*i.e.* continuous white light): whereas the VC embryos were largely quiescent (post-diapause embryos, hatching in suitable hatching conditions), BY and especially TK may have been partially in diapause. Light exposure may have a different effect on diapausing versus post-diapausing embryos, contributing to the differences observed between the different samples. Our limited set of strains did not cover the entire biodiversity within the genus *Artemia* and did not allow unraveling the possible role of the factors mentioned above. Nevertheless, in spite of the numerous factors which may interfere when working with samples of different strains and with different history, the inter-strain differences observed in our study were only of quantitative nature, suggesting that the interaction of light with the *Artemia* hatching process is consistent throughout the genus.

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Chapter 7

Effect of light colors in combination with hydrogen peroxide or nitric oxide on the hatchability and diapause termination of *Artemia* cysts from different geographical localities

Abstract

The present study aimed to investigate the effect of exposure to chemicals (H_2O_2 or NO) in combination with light on the hatching of cysts of one strain of *Artemia franciscana* (Vinh Chau, Vietnam), which was largely out of diapause, and two strains of parthenogenetic *Artemia*: Bolshoye Yarovoye (Russia) and Tuz Lake (Kazakhstan). The latter sample was predominantly in diapause whereas the former contained a mixture of diapausing and quiescent cysts, as shown by their hatching in standard conditions. Three different light colours (red, blue and white) were used.

Our results revealed that light has a high effect in stimulating hatching, but differently when different colours were used. Exposure to H_2O_2 or NO also enhanced hatching percentage, but the effect of H_2O_2 was more prominent. When light was provided together with H_2O_2 or NO, sometimes a synergistic effect was found, with the effect of both factors supplied together higher than the sum of the effects of the individual factors. This was the case in the Tuz Lake strain when red light was provided with NO, or blue/white light with H_2O_2. Sometimes a compensatory effect was found, with the effect of both factors supplied together being lower than the sum of the effect of each individual factor. This was generally the case for the Vinh Chau and Bolshoye Yarovoye strains, when supplying NO together with light of any colour. In most other cases the effects of both factors were additive.

It is concluded that the two chemicals act differently on the hatching process in the *Artemia* samples studied; this is probably related to their state of diapause, but may also be related to other strain-specific differences. Differences in cyst hatching patterns as influenced by light and chemicals could become the basis for the characterization of cysts relative to their diapause and post-diapause state.

7.1. Introduction

Artemia cysts undergo diapause, a dormant stage where metabolism and development stops and that is tolerant to stress (Drinkwater and Clegg, 1991). The role of diapause in dispersal can be important in crustaceans (Van Stappen, 1996; Saygi, 2003). The diapause stage is genetically programmed to pass the forthcoming seasonal adverse conditions, *e.g.* cold harsh winters or dry intolerable seasons (Nambu et al., 2009). Under unfavourable environmental conditions, female *Artemia* release cysts in a diapause state instead of free swimming nauplii. One of the key advantages of *Artemia* as live feed is its ability to produce these encysted embryos that can be stored for a long period without losing their hatching ability.

For a successful diapause termination and hatching, the *Artemia* diapause has to be broken by (an) appropriate environmental cue(s). In nature, this is achieved by various means (Lavens and Sorgeloos, 1987; Brendonck, 1996), most commonly by dehydration and/or hibernation (Lavens and Sorgeloos, 1987; Drinkwater and Clegg, 1991). The effectiveness of each of these processes in terminating diapause varies, depending on the *Artemia* strain in consideration. Termination of diapause leads to another type of "latent life", called quiescence. This latter type of cryptobiosis is under the control of exogenous factors, meaning that metabolic processes are resumed as soon as the cysts are brought in favourable external (hatching) conditions (Drinkwater and Crowe, 1987). It is important to take into consideration that the mechanisms for the induction of the state of diapause, as well as the internal mechanism of its deactivation process, are not yet completely understood.

Diapause cysts will not hatch under normal hatching conditions unless the diapause state is terminated by physical or chemical stimuli. In this regard, for the user of *Artemia* cysts, various

processes have been proven successful in terminating diapause (review by Lavens and Sorgeloos, 1987). A hydrogen peroxide-treatment, for example, was applied with variable success by several authors, but without much uniformity in methodology (Mathias, 1937; Bogatova and Shmakova, 1980; Bogatova and Erofeeva, 1985). In additional studies (Lavens et al., 1986; Vu Do Quynh et al., 1987; Van Stappen et al., 1998) different strains, and various H_2O_2 concentrations and treatment periods were used: the effect of the treatment was variable as well, but despite the variability, a considerable portion of diapausing cysts could be activated in all cases. Robbins et al. (2010) compared the ability of hydrogen peroxide and nitric oxide (NO) to trigger development in both quiescent and diapause embryos of *Artemia*. However, the mechanism by which this empirical approach with hydrogen peroxide actually induces the effect is completely unknown (Robbins et al., 2010). Moreover, the failure of NO to break the diapause state was attributed by these authors to its hypothesized role in solely enhancing post-diapause embryo development by driving changes in cell structure and gene expression.

Our previous study has shown that light colour, timing and duration of exposure have a marked effect on the hatchability of *Artemia* cysts (chapter 6), with a difference in light sensitivity of cysts of different geographical origin (Vanhaecke et al., 1981), although the effect on diapause termination itself versus the effect on post-diapause cyst hatching metabolism remains to be explored (Pancella and Stross, 1963; Bishop, 1967; Hempel-Zawitkowska, 1970; Sorgeloos, 1973; Takahashi, 1975, 1977; Mitchell, 1990; Horiguchi et al., 2009; Nambu et al., 2008, 2009).

Moreover, earlier work indicates that branchiopod embryos are activated by a broad spectrum of wavelengths, from ultraviolet (UV; 395 nm) to red (660 nm) (Kashiyama *et al*., 2010), a finding that suggests the involvement of different optical pigments in photoreception. The evolutionary

relevance of responses to light and other cues is mentioned by different authors (Murugan and Dumont 1995, Cáceres et al., 2007; Kashiyama et al., 2010).

Furthermore, it is still unclear whether the increased hatching is an effect of diapause termination or related to the hatching metabolism itself. In addition, literature data do not provide reliable information, and relatively little is known about the combined effect of light and chemicals on hatching and diapause termination.

Our previous study on the effect of different aspects of illumination on the hatching of cysts has shown that a critical amount of light energy has to be administered to achieve maximal hatching (chapter 6). In the present chapter, we aimed to investigate whether H_2O_2 and NO have any beneficial effect on *Artemia* cyst hatching percentage and diapause deactivation. More specifically, we wanted to find out if chemicals can have additional effects beyond those observed with light, and if they can fully or partially replace the effect of light, provided in various conditions, during cyst incubation. By using samples of different *Artemia* strains (and species), we attempted to gain more insight in the strain-specificity of these effects.

For this purpose, different factors were considered: different light colour with different light intensities and different wavelengths, and different duration of hatching incubation (24 and 48 h) in combination with two different chemicals. Hatching was used as criterion to detect and quantify the combined and the individual effects of light and chemicals on *Artemia* cysts.

7.2. Materials and methods

7.2.1. Experimental design

The experiments were conducted in order to determine the combined and the individual effect of light and of exposure of chemicals (H_2O_2 or NO) on *Artemia* cysts hatching percentage for either

diapausing or non-diapausing cysts. For each strain, the two different chemicals and light exposure were tested over a hatching incubation period of 24 and 48 h. Continuous darkness and continuous light were included as negative and positive controls, respectively.

7.2.2. Cyst samples

Experiments were performed with three strains of dried *Artemia* cysts: one was a commercial dry sample of the bisexual species *Artemia franciscana* Kellogg 1906 originating from Vinh Chau (VC) salt fields, Vietnam (ARC code 1742), and collected in 2009. The VC strain had resulted from an initial inoculation of San Francisco Bay *A. franciscana* in the mid-1980s and subsequent re-inoculation every dry season since then. A second sample was parthenogenetic *Artemia* from Tuz Lake (TK), Kazakhstan (ARC code 1761, collected in 2005), and the third sample was parthenogenetic *Artemia* from Bolshoye Yarovoye (BY), Siberia, Russia (ARC code 1758, collected in 2005). All samples had been stored at + 4 °C since their arrival at the Laboratory of Aquaculture & *Artemia* Reference Center, which is the current procedure to ensure maximal viability and hatchability of the cysts, even in dried form (Lavens and Sorgeloos, 1996). Values of hatching in standard hatching conditions in a first screening test have been mentioned in chapter 6 (section 6.2.1). Based on these results, the TK parthenogenetic strain was considered as being largely in diapause, based on its very low hatching values after prolonged hatching incubation period. The VC sample was considered as being largely in quiescent stage based on its relatively high hatching. The BY strain, with moderate hatching values, was considered to be partially in diapause, partially in quiescence.

7.2.3. Preparation of hydrogen peroxide (H_2O_2) and nitric oxide (NO) stock solution

7.2.3.1. Determination of optimal concentration of chemicals

For the two chemicals, the concentrations resulting in maximal hatching had been determined in preliminary screening tests (results not shown) using different concentrations and standard hatching conditions for the three *Artemia* strains: 0.5, 1, 2, 3, 6, 9, 12 and 36 mg L^{-1} for H_2O_2 and 0.015, 0.03, 0.06, 0.12, 0.18, 0.24, 0.3, 0.6, 1.2 and 1.8 mg L^{-1} for NO. Out of these, for H_2O_2, 6 mg L^{-1} (0.18 mmol L^{-1}) and for nitric oxide 0.03 mg L^{-1} (0.17 µmol L^{-1}) had been proven to be most efficient in enhancing hatching, and thus these concentrations were selected for further experiments.

7.2.3.2. Preparation of solutions for experiments

The H_2O_2 solution was prepared just before use by taking 1 mL from a commercial stock solution of 30 % w/w H_2O_2 (Sigma-Aldrich, St Louis, MO, USA) and adding 29 mL of Instant Ocean® artificial seawater (32 g L^{-1}). We took 300 µL of this diluted solution and added it to 500 mL of Instant Ocean® seawater (32 g L^{-1}). From this last solution 25 mL with a concentration of 0.18 mmol L^{-1} H_2O_2 were then transferred to falcon tubes together with 0.05 g of *Artemia* cysts. For NO preparation, 8.811 mg of NO donor 3-(2-hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine (Papa NONOate; half-life 15 min) was dissolved in 200 mL of Instant Ocean® seawater (32 g L^{-1}). From this dilution 50 µL with a concentration of 0.17 µmol L^{-1} NO were transferred to falcon tubes containing 25 mL of Instant Ocean® seawater together with 0.05 g of *Artemia* cysts.

7.2.4. Treatment of cysts by NO and H_2O_2 in combination with different light colours

All hatching experiments were done in triplicates. Cysts (0.05 g) were incubated for hatching in sterile 50 mL screw-cap falcon tubes containing 25 mL of Instant Ocean® artificial seawater of 32 g L^{-1} salinity supplemented with either 0.17 µmol L^{-1} NO, or with 0.18 mmol L^{-1} H_2O_2 as described above, and kept in suspension on a rotator at 4 cycles per min to prevent clogging and sedimentation of the cysts, in a room at 28 ± 1 °C.

Fluorescent lamps (Philips TL-D 18W, SLV) generating red (600 – 700 nm), blue (400 – 500 nm) or white (400 – 700 nm) light within the visible spectrum, according to the manufacturer's specification, were positioned 20 cm above the hatching set-up. The different light treatments were shaded from each other. The light intensity (expressed as µE·m^{-2} s^{-1}) reaching the surface of the hatching medium was measured with a light sensor of a light meter (LI-Cor sensor-190) and was 27, 24 and 22 µE·m^{-2} s^{-1} for red, blue and white light, respectively. In all cases and during the darkness incubation, sampling and manipulation of cysts was always taking place under red light.

7.2.5. Determination of hatching percentage (H %)

After 24 h and 48 h of hatching incubation, six subsamples of 250 µL each were taken from each falcon tube with a micropipette and placed in a small vial. Nauplii were fixed by adding a few drops of lugol solution and tap water. The nauplii as well as the umbrellae were counted under the microscope. The unhatched cysts were subsequently decapsulated by adding a few drops of NaOCl and NaOH solution to each vial, resulting in decapsulation (Bruggeman et al., 1980), and the orange coloured embryos were counted.

The hatching percentage was calculated as follows (modified from Van Stappen, 1996): the embryos in the umbrella stage were considered as having reacted to the light and/or chemical triggers, and were thus included in the calculation of the hatching percentage:

H % = N + U/ (N + U + E) x 100, where N = number of nauplii, U = number of umbrellae, E = number of embryos.

The mean hatching value per falcon tube was recorded and the overall mean hatching percentage and standard deviation for the three replicate falcon tubes were calculated for the 24 and 48 h hatching incubation period.

7.2.6. Statistical analysis

To evaluate the effect of different light colours in combination with chemicals on hatching percentage for the three strains of *Artemia* after incubation periods of 24 and 48 h, a three-way ANOVA model was used in SAS (SAS version 9.4, SAS Institute, Cary, NC) (Verbeke, G. and Molenberghs, G. 2000). Hatching percentage data (the dependent variable) were normalized using the arcsin-square-root transformation prior to statistical analysis, while only non-transformed means are presented in the figures. In a first step, strain, light colour and incubation time were set as fixed effects in fitting the model to the control data (continuous darkness and continuous light). In the second step, for each incubation period separately (24 or 48 h), strain (TK, BY and VC), light colour (red, blue and white) and treatment (continuous darkness, continuous light, continuous darkness plus chemicals and continuous light plus chemicals) were set as fixed effects (independent variables). All interactions between these variables were assessed for significance ($P < 0.05$). All pairwise comparisons were done with a Tukey's post hoc adjustment method for multiple testing (Sherri, 2012). Both the H_2O_2 as well as the NO trials were analyzed separately.

In the third step, for each strain (TK, BY and VC), each incubation period separately (24 or 48 h) and each light colour (red, blue and white), the pairwise interaction between exposure to light and exposure to a chemical on hatching percentage was assessed for significance ($P < 0.05$) using a two-way ANOVA model (SPSS, version 24). All pairwise comparisons were done with a Tukey test to detect the significant differences. Both the H_2O_2 as well as the NO trials were analyzed separately.

7.3. Results

7.3.1. The effect of different light colours in combination with H_2O_2 on the hatching of Artemia cysts

The hatching percentage of cysts exposed to 24 h continuous white light in combination with H_2O_2 was highest for VC (91.2 %), moderate for BY (65.5 %) and lowest for TK (59.8 %) (Fig. 7.1. E, C and A, respectively). The 24 h values were lower when using continuous blue light in combination with H_2O_2 (84.6, 59.6 and 53.7 % for VC, BY and TK, respectively) than with white light, and when using red light in combination with H_2O_2 they were the lowest (53.9, 47.4 and 29.7 %, respectively). In continuous darkness, hatching was lower than under any light source plus H_2O_2 (18.2, 6.5 and 3.5, respectively, after 24 h) (Fig. 7.1 E, C and A).

In continuous darkness plus chemicals, a very high increase in hatching was observed compared to hatching in darkness for 24 h: for the TK strain hatching increased more than six fold (from 3.5 to 23.6 %). The same was also observed with the BY strain (from 6.5 to 42.7 %), while for VC there was just a minimal increase (from 23.6 to 24.3 %) (Fig. 7.1 A, C and E, respectively). For each strain, continuous light exposure (red, blue and white light) resulted in hatching improvement compared to darkness after 24 h of incubation. For TK and BY, there were no differences in H %

under continuous blue and white light (without H_2O_2) compared to darkness + H_2O_2, while continuous red light resulted in lower hatching. In contrast, in VC a lower hatching was obtained in darkness + H_2O_2, compared to continuous light (without H_2O_2) of any colour (Fig. 7.1 A, B and C, D).

If the cysts were subjected to different light colours + H_2O_2 the hatching percentage after 24 h of incubation resulted in a further increase compared to darkness, with a hatching percentage of 29.8, 53.6 and 59.8 % for the TK strain using red, blue and white light, respectively. This was also observed for the other two strains with an increase in hatching up to 47.4, 59.6 and 65.5 % for BY, and up to 53.9, 84.6 and 91.2 % for VC cysts (Fig. 7.1. A, C and E).

The statistical analysis using three-way ANOVA of the control hatching values obtained with continuous light of different colours and continuous darkness showed that there was no significant three-way interaction ($P > 0.05$) between the period of incubation of cysts for hatching (24 or 48 h), strain and light colour (Table 7.1). Moreover, there were no significant pairwise interactions ($P > 0.05$) (Table 7.1).

Three-way ANOVA analysis of the 24 h hatching values, obtained in treatments with H_2O_2 in combination with light exposure, showed a significant effect ($P < 0.0001$) of strain, light colour and treatment with H_2O_2 on hatching (Table 7.2). Also all pairwise interactions were significant. This was also the case for the 48 h hatching values (Table 7.2). This indicates that the effect of the additional H_2O_2 treatment on hatching depends on light colour and on strain whether for 24 or 48 h. Overall the hatching percentage was significantly different under the different light colours, between the different strains and with versus without H_2O_2. In addition, analysis of pairwise interaction using two-way ANOVA between exposure to light versus exposure to H_2O_2 showed a significant interactive effect ($P < 0.05$) on the hatching values after 24 h for the TK and BY strains

with blue or white light, whereas for the VC strain, the interaction was not significant ($P > 0.05$) (Fig. 7.2 and Table 7.3). With red light on the other hand interaction was non-significant in TK and significant in BY and VC strains. As for 48 h values, two-way ANOVA showed the same results as for the 24 h values, except for the fact that in red light there was no significant interaction for any strain (Table 7.3 and Fig. 11.1 in appendix E).

Table 7.1: Statistics of the fixed effects of hatching incubation time, strain, light colour, and their interactions, on hatching values in the controls (continuous light and darkness). Num.DF = numerator degrees of freedom; Den.DF = denominator degrees of freedom.

Tests of fixed effects				
Effect	**Num. DF**	**Den. DF**	**F-value**	**P-value**
Light colour	2	90	4.9	0.0092
Strain	2	90	35.5	<.0001
Light colour*Strain	4	90	0.1	0.9915
Incubation time	1	90	1.9	0.1728
Light colour*Incubation time	2	90	0.00	0.9989
Strain*Incubation time	2	90	0.4	0.7039
Light colour*Strain*Incubation time	4	90	0.03	0.9987

Table 7.2: Statistics of the fixed effects of strain, light colour and H_2O_2 treatment, and their interactions, on hatching values after 24 and 48 h when combining H_2O_2 with light exposure. Num.DF = numerator degrees of freedom; Den.DF = denominator degrees of freedom.

Tests of fixed effects								
Time	**24 h**				**48 h**			
Effect	**Num. DF**	**Den. DF**	**F-value**	**P-value**	**Num. DF**	**Den. DF**	**F-value**	**P-value**
Light colour	2	72	270.86	<.0001	2	72	352.79	<.0001
Strain	2	72	816.66	<.0001	2	72	1557.43	<.0001
Light colour*Strain	4	72	11.59	<.0001	4	72	6.92	<.0001
H_2O_2 treatment	3	72	1881.46	<.0001	3	72	2472.11	<.0001
Light* H_2O_2 treatment	6	72	90.95	<.0001	6	72	118.78	<.0001
Strain*H_2O_2 treatment	6	72	180.70	<.0001	6	72	276.80	<.0001
Light colour*Strain* H_2O_2 treatment	12	72	4.85	<.0001	12	72	5.36	<.0001

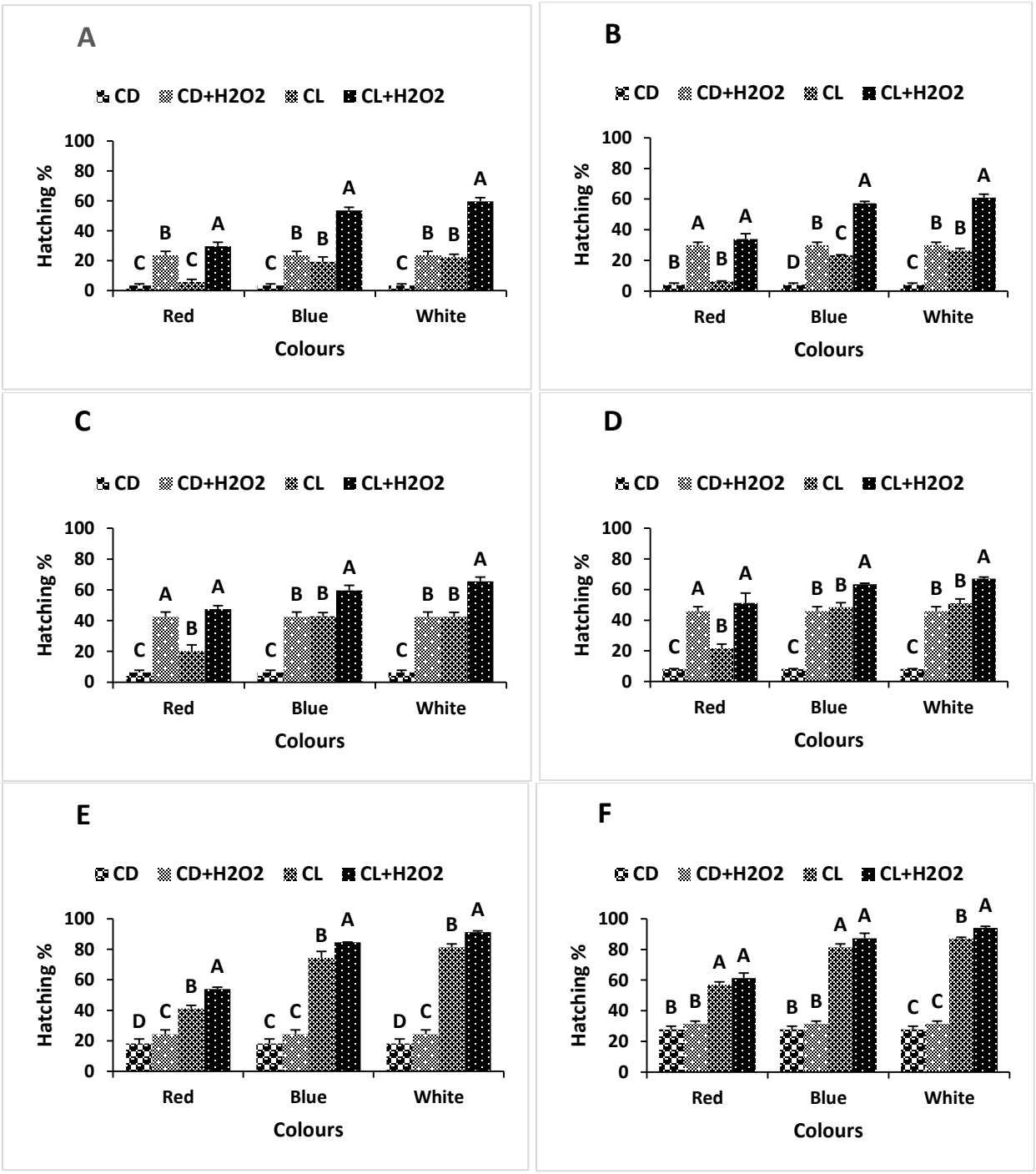

Figure 7.1: Hatching percentage of cysts of **TK (**top), **BY** (middle) and **VC** (below) *Artemia* strains exposed to different treatments: continuous darkness (CD), continuous light (CL), continuous darkness in combination with H_2O_2 (CD + H_2O_2) and continuous light in combination with H_2O_2 (CL + H_2O_2), and incubated for 24 h and 48 h (**A, B**), (**C, D**) and (**E, F**) for TK, BY and VC respectively. Superscripts on bars in each figure correspond with significant differences between the different treatments for each light colour (red, blue and white). Data are mean value (n = 3) ± standard deviation. Significance level was set at $P < 0.05$. TK = Tuz, Kazakhstan. BY = Bolshoye Yarovoye, Russia. VC = Vinh Chau, Vietnam.

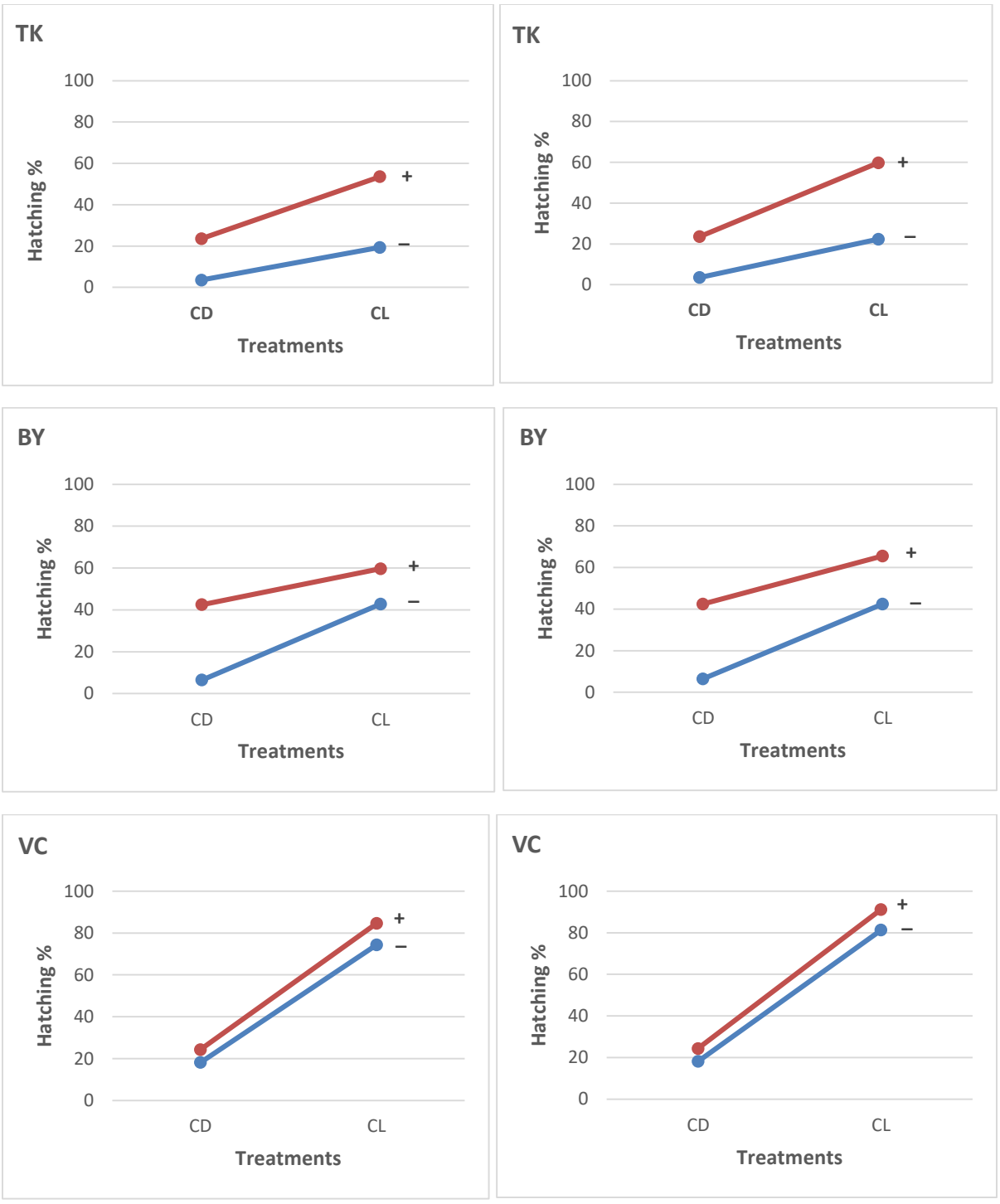

Figure 7.2: Hatching percentage of cysts of **TK**, **BY** and **VC** *Artemia* strains exposed to continuous darkness (CD) and continuous light (CL) in combination with H_2O_2 (+) or without H_2O_2 (-) and incubated for 24 h. The lines in each figure correspond to treatments with blue light (left) and white light (right). Data are mean value (n = 3). TK = Tuz, Kazakhstan. BY = Bolshoye Yarovoye, Russia. VC = Vinh Chau, Vietnam.

Table 7.3: Pairwise interaction (two-way ANOVA) between exposure to light and exposure to chemicals (H_2O_2 and NO) and their effects on hatching % after 24 and 48 h with different light colours (red, blue and white) for the three strains TK, BY and VC. *P*-values of significant interactions are in bold; SY = synergistic effect; A = additive effect; C = compensatory effect. Significance level was set at $P < 0.05$.

		***P*-values**											
Light Colours		**Red light**				**Blue light**				**White light**			
Chemicals		**H_2O_2**		**NO**		**H_2O_2**		**NO**		**H_2O_2**		**NO**	
Incubation time		**24 h**	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**
TK	***P*-value**	0.159	0,469	**0.001**	**0.021**	**0.001**	**0.000**	0.535	0.196	**0.000**	**0.002**	0.119	0.207
	Effect	A	A	SY	SY	SY	SY	A	A	SY	SY	A	A
BY	***P*-value**	**0.028**	0.101	0.345	0.185	**0.000**	**0.000**	**0.000**	**0.000**	**0.003**	**0.000**	**0.006**	**0.000**
	Effect	C	A	A	A	C	C	C	C	C	C	C	C
VC	***P*-value**	**0.024**	0.783	**0.016**	**0.000**	0.231	0.430	**0.012**	**0.041**	0.154	0.098	**0.002**	**0.000**
	Effect	C	A	C	C	A	A	C	C	A	A	C	C

7.3.2. The effect of different light colours in combination with NO on the hatching of Artemia cysts

In the second part of the experiment, the exposure of the cysts for 24 h of total darkness + NO resulted in a significant increase ($P < 0.05$) in hatching % up to 18.4, 32.2 and 33.8 % for TK, BY and VC, respectively, compared to total darkness (3.6, 6.5 and 18.2 %). If NO was used in combination with light, the hatching percentage after 24 h of incubation resulted in a further significant increase ($P < 0.05$) to 28.9, 35.6 and 40.4 % for TK using continuous red, blue and white light respectively. This was also observed for the other two strains with a significant increase in hatching % up to 43.0, 48.9 and 57.4 for BY and 49.7, 79.9 and 86.7 % for VC cysts, compared to continuous darkness or darkness plus NO (Fig. 7.3).

The statistical analysis using three-way ANOVA of the control values (continuous light of different colours and continuous darkness) showed the same trend as the control values in the test using H_2O_2, and the hatching incubation time (24 or 48 h), as well as strain and light colour, had no significant interaction effect ($P > 0.05$) on the hatching values (Table 7.4).

Three-way ANOVA analysis of the 24 h hatching values, obtained in treatments with NO in combination with light exposure, showed a significant effect ($P < 0.0001$) of strain, light colour and treatment with chemical (Table 7.5). Also all pairwise interactions were significant. This was also the case for the 48 h hatching values (Table 7.5). This indicates that, similarly to when using H_2O_2, the effect of the NO treatment depended on the light colour and on the strain, both at 24 and 48 h. Overall the hatching % was significantly different under the different light colours, between the different strains and with versus without NO. In addition, analysis of pairwise interaction using two-way ANOVA between exposure to light versus exposure to NO showed a significant interactive effect ($P < 0.05$) on the hatching values after 24 h of the BY and VC cysts with blue

and white light, whereas for the TK strain the interaction was not significant (Fig. 7.4 and Table 7.3). With red light, interaction was non-significant in BY and significant in TK and VC strains. All this was exactly the same for the 48 h values (Table 7.3 and Fig. 11.2 in appendix E).

Table 7.4: Statistics of the fixed effects of hatching incubation time, strain, light colour, and their interactions, on hatching values in the controls (continuous light and darkness). Num.DF = numerator degrees of freedom; Den.DF = denominator degrees of freedom.

Tests of fixed effects				
Effect	**Num. DF**	**Den. DF**	**F-value**	**P-value**
Light colour	2	90	4.9	0.0092
Strain	2	90	35.5	<.0001
Light colour*Strain	4	90	0.1	0.9915
Incubation time	1	90	1.9	0.1728
Light colour*Incubation time	2	90	0.00	0.9989
Strain*Incubation time	2	90	0.4	0.7039
Light colour*Strain*Incubation time	4	90	0.03	0.9987

Table 7.5: Statistics of the fixed effects of strain, light colour and NO treatment, and their interactions, on hatching values after 24 and 48 h when combining NO with light exposure. Num.DF = numerator degrees of freedom; Den.DF = denominator degrees of freedom.

Tests of fixed effects								
Time	**24 h**				**48 h**			
Effect	**Num. DF**	**Den. DF**	**F-value**	**P-value**	**Num. DF**	**Den. DF**	**F-value**	**P-value**
Light colour	2	72	234.5	<.0001	2	72	261.1	<.0001
Strain	2	72	1421.3	<.0001	2	72	2010.1	<.0001
Light colour*Strain	4	72	19.7	<.0001	4	72	11.9	<.0001
NO treatment	3	72	1696.3	<.0001	3	72	1792.4	<.0001
Light colour*NO treatment	6	72	85.3	<.0001	6	72	92.9	<.0001
Strain*NO treatment	6	72	98.3	<.0001	6	72	124.03	<.0001
Light colour*Strain*NO treatment	12	72	8.0	<.0001	12	72	8.8	<.0001

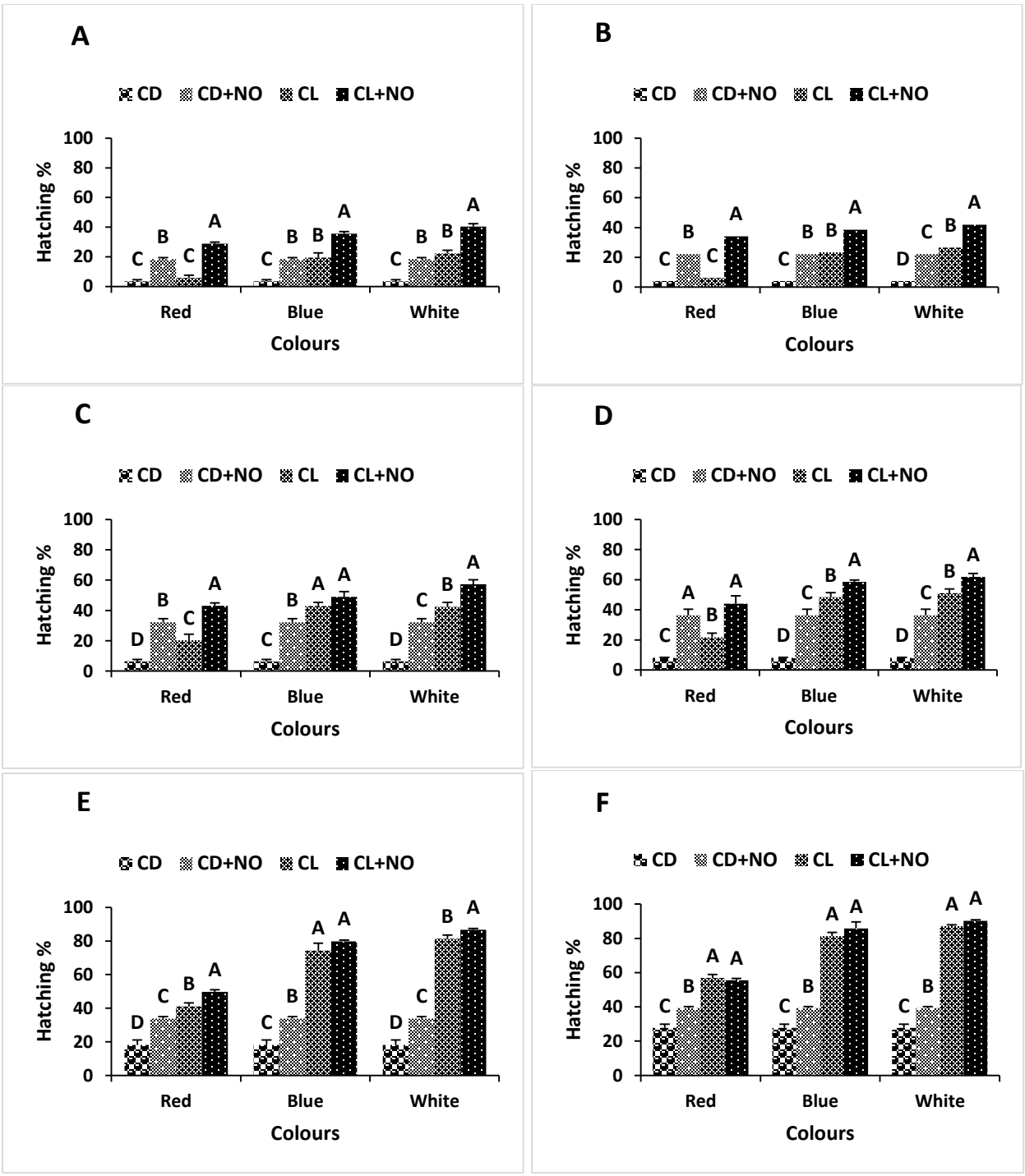

Figure 7.3: Hatching percentage of cysts of **TK** (top), **BY** (middle) and **VC** (below) *Artemia* strains exposed to different treatments: continuous darkness (CD), continuous light (CL), continuous darkness in combination with NO (CD + NO) and continuous light in combination with NO (CL + NO), and incubated for 24 h and 48 h (**A, B**), (**C, D**) and (**E, F**) for TK, BY and VC respectively. Superscripts on bars in each figure correspond with significant differences between the different treatments for each light colour (red, blue and white. Data are mean value (n = 3) ± standard deviation. Significance level was set at $P < 0.05$. TK = Tuz, Kazakhstan. BY = Bolshoye Yarovoye, Russia. VC = Vinh Chau, Vietnam.

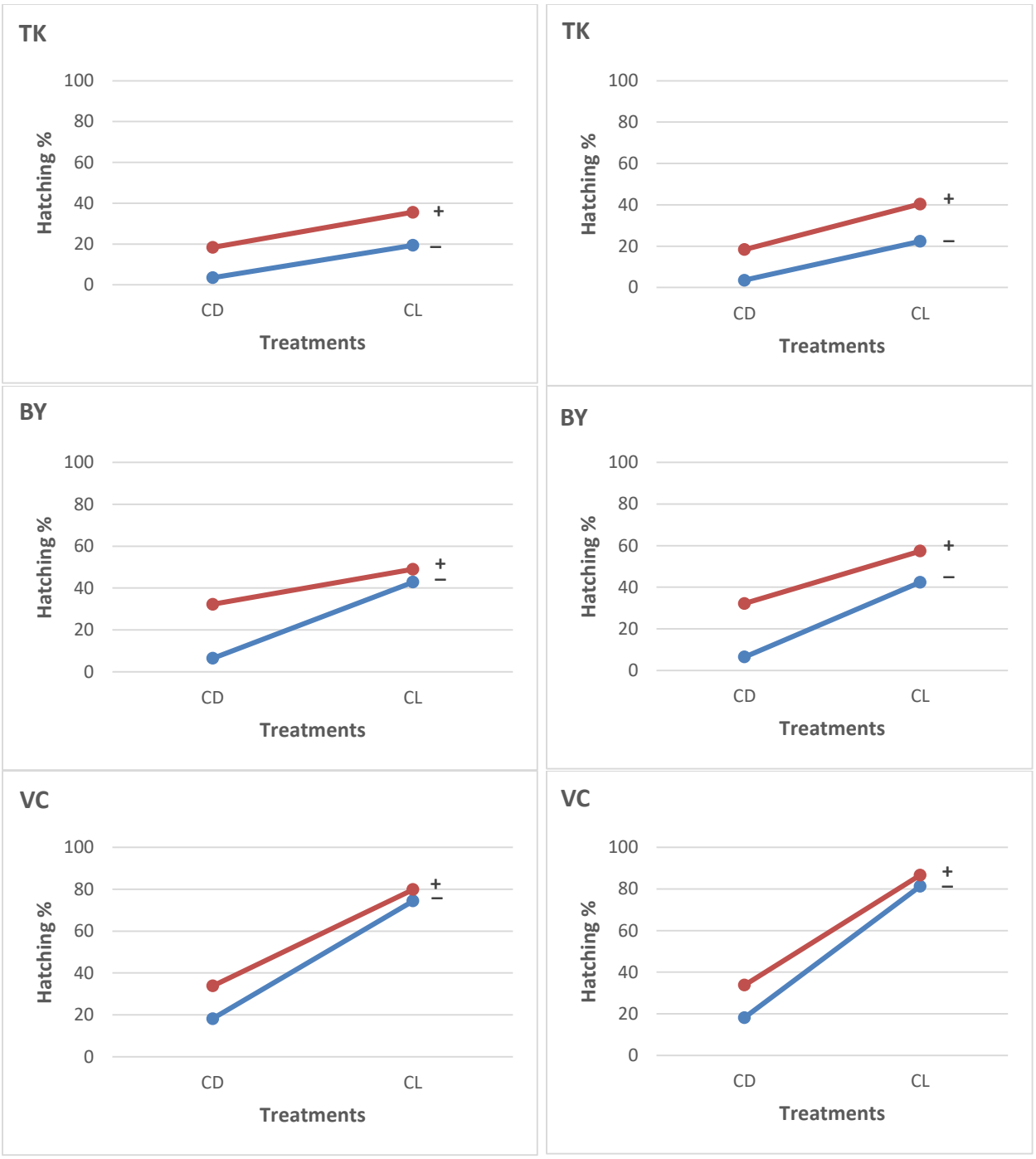

Figure 7.4: Hatching percentage of cysts of **TK**, **BY** and **VC** *Artemia* strains exposed to continuous darkness (CD) and continuous light (CL) in combination with NO (+) or without NO (-) and incubated for 24 h. The lines in each figure correspond to treatments with blue light (left) and white light (right). Data are mean value (n = 3). TK = Tuz, Kazakhstan. BY = Bolshoye Yarovoye, Russia. VC = Vinh Chau, Vietnam.

7.4. Discussion

Diapause is a condition of metabolic arrest where *Artemia* embryonic development stops. To better understand the effects of H_2O_2 and NO in combination with light on hatching of diapause and post-diapause (quiescent) cysts, we used three light colours with different intensities and wavelengths, two different chemicals, and cysts in different states of diapause. Moreover, to take into consideration the possibility that sensitivity to these treatments is strain-dependent, we used *Artemia* cysts from three different geographical regions.

As expected, light exposure in combination with H_2O_2 or NO indeed can significantly enhance the hatching percentage compared to incubation in darkness or in the absence of the two chemicals. This is in agreement with the previous results on the effects of different light parameters on hatching (chapter 6), and with literature results (Kashiyama et al., 2010; Van Stappen et al., 1998; Saygi, 2003; Robbins et al., 2010). H_2O_2 and NO are reported to influence physiological and developmental processes in many organisms (Bian et al., 2006; Stone and Yang, 2006; Bright et al., 2006; Giorgio et al., 2007; Zhang et al., 2007; Covarrubias et al., 2008; Neill et al., 2008; Forman et al., 2008; Zhao and Shi, 2009), including the germination of seeds, biological structures that share characteristics with *Artemia* cysts (for review see Robbins et al., 2010 and references therein).

H_2O_2 is a form of reactive oxygen species (ROS) generated during oxidative stress (Neill et al., 2002b). Along with its derived reactive oxygen form, it can react with various cellular targets (Bartosz, 1997; Neill et al., 2002b). The oxidative properties of H_2O_2 suggest how H_2O_2 functions as signaling molecules capable of terminating diapause and promoting post-diapause development. Presumably, H_2O_2 works by either activating or inhibiting regulatory, metabolic and structural proteins in the cysts (Robbins et al., 2010), leading to a condition in which the cysts sense the

environment as being favourable for survival and thus terminate diapause and proceed into the development process.

In this experiment, we found that the hatching of TK, BY and VC cysts was significantly higher under any light exposure in combination with H_2O_2 than when these factors were not combined. Pairwise interaction between exposure to blue or white light and exposure to H_2O_2 showed a significant effect on the hatching % of TK and BY strains after 24 h. A synergistic effect was obtained for the TK strain: exposure to blue light and H_2O_2 together increased the hatching % with 50.2 % as compared to hatching in darkness without chemical, which was more than the sum of the effects of exposure to blue light and to H_2O_2 alone (15.9 and 20.1 % increase, respectively). For BY, providing both blue light and H_2O_2 resulted only in a compensatory effect: exposure to blue light increased the hatching % with 36.2 % as compared to darkness; exposure to H_2O_2 with 36.3 %, whereas exposure to both factors together increased the hatching % with 51.1 %, which was lower than the sum of the effects of each factor separately. For the VC strain the interaction between exposure to blue light and to the chemical was not significant; the combined effect of the two factors together was similar to the sum of the individual effects (63.1 % increase when exposing to blue light, 6.1 % increase when exposing to H_2O_2, 73.0 % when exposing to both factors). This indicates that providing both factors had an additive effect on hatching of cysts for this *Artemia* strain. The effect for the 48 h values was the same as for the 24 h values with blue and white light. With red light a significant compensatory effect for BY and VC strains was observed in 24 h values and an additive effect in all other cases.

Also NO has been found to be effective in triggering the *Artemia* cyst hatching mechanism. NO is a free radical signaling molecule typically converted rapidly into NO_3^- and NO_2^- by nitrogen dioxide (Neill et al., 2003; Forman et al., 2008). NO may advance cyst development by acting as

a reactive nitrogen species which drives the formation of NO-metallo linkages in haem-containing proteins (Villalobo, 2006; Forman et al., 2008). Typically, NO is applied to most organisms via a NO-donor; that is a substance which releases NO, sometimes after passage into cells. The speed at which the release happens is reflected by the half life time, which is the time during which the donor releases half of the concentration of nitric oxide (Neill et al., 2003). NO rapidly crosses biological membranes and triggers various processes in a short period of time (Beligni and Lamattina, 2001).

In the quiescent VC cysts, a compensatory effect was found between light of any colour and NO exposure, both in 24 and 48 h hatching values. In the TK cysts (which were predominantly in diapause) a synergistic effect was found only when red light was used, and an additive effect was found for blue and white light. In BY, finally, both blue and white light resulted in a compensatory effect with NO exposure.

Although both Robbins et al. (2010) and our study deal with diapause and post-diapause deactivation in *Artemia* cysts using chemicals, the former study focused on assessing the role of NO versus that of H_2O_2, whereas our study focused more on the effect of chemicals versus that of light, and the combination of both, using three different light colours. Robbins et al. (2010) used cysts from two strains, Bolshoye Yarovoye and Great Salt Lake, which were predominantly in diapause (hatching 25 % and 15 %, respectively). Our results corroborate their findings that NO and H_2O_2, supplied during the first 4 h of hatching incubation, both initiated development processes that continued when the chemical was washed away (Robbins et al., 2010). In our study, although NO was substantially less effective than H_2O_2 at enhancing hatching, it induced considerable hatching, especially in the TK strain, which was in diapause state.

Because light and chemicals must penetrate the protective egg shell (including the cuticular membranes) to influence the photochemical receptive molecules inside the embryo, the egg shell is thought to influence the sensitivity and the response time of resting eggs to light and chemical exposure (Clegg et al., 1996). We found that the effect of light was strain-specific. Chorion thickness, which varies between strains (Vanhaecke *et al*., 1981), could be one of the factors, as a thinner chorion would allow more light to pass through. Among the three strains used, the VC cysts have the thinnest chorion (chapter 6) and they also gave the highest hatching percentages. Likewise, the effect of the chemicals could also be affected by chorion thickness. Differences in responsiveness to NO and H_2O_2 treatment may be genetic, but they might also be due to environmental factors before and during cyst harvesting, as well as during processing and storage (Van Stappen et al., 1998; Robbins et al., 2010). Moreover, the effects of environmental conditions during embryo formation on the "depth" of developmental arrest and ease of activation have been documented in other organisms (Drinkwater and Clegg, 1991).

We conclude that H_2O_2 and NO exerted a strong effect on hatching and terminating the diapause state of *Artemia* cysts but the effect of H_2O_2 was more prominent. The differences observed between the different strains, may also be related to environmental factors or genotypical differences in sensitivity of cysts of different geographical origin. Our results may contribute to better understanding of the combined effects of light and chemicals, and may provide new tools to terminate diapause and enhance hatching of post-diapause cysts. For future work, it might be interesting to conduct research with more combinations of different light conditions and chemicals, including different duration and timing of exposure to know to what extent H_2O_2 or NO could replace light during *Artemia* cysts hatching incubation.

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The authors are grateful to the Ministry of Higher Education of the Libyan Government who supported this study through a doctoral grant to the first author. We are also grateful to all the staff of Laboratory of Aquaculture & *Artemia* Reference Center, Department of Animal Sciences and Aquatic Ecology, Faculty of Bioscience Engineering, Ghent University for their help, support and guidance during the lab work of the experiments.

Chapter 8

General discussion, conclusions and future perspectives

8.1. General discussion

Generally speaking, *Artemia* nauplii, as a live food, are still required in the larviculture of many aquatic species despite the availability of micro-diets. In fact worldwide, hatcheries still consume more than 3000 tonnes of dry cysts annually.

Hence there is a general interest in the quality of cysts in the aquaculture sector. In this thesis various quality aspects of cysts and emerging nauplii were investigated. The results of the experiments are reviewed here with a special emphasis on the importance for the aquaculture industry. Fig 8.1 presents an overview of the linkage between the different chapters of the thesis.

8.1.1. Effect of successive H/D cycles on the quality of cysts and of the emerging nauplii used as live food

There was an aggravating effect on the quality of the cysts and the emerging nauplii as the number of cycles increased from one to three, and as the hydration period, preceding dehydration, was lengthened from 2 to 4 h. A loss of hatching, in the range of 10 – 12 % and 50 – 60 % as a result of multiple H/D cycles of 2 and 4 h respectively, was observed relative to the control after one month of storage. A linear mixed model (LMM) was used to evaluate the effect of different H/D cycles on hatching % as evaluated after different storage periods. This showed that in both strains a 4 h hydration cycle compromised hatching significantly ($P < 0.0001$) relative to 2 h hydration (Table 9.1 and Fig. 9.1 in appendix C). This lower hatching percentage corroborates earlier findings by *e.g.* Morris (1971) demonstrating that the number of hatched cysts eventually dropped very sharply after three cycles of 6-h hydration, presumably owing to a depletion of energy reserves. In addition, other factors such as cyst storage conditions can potentially affect cyst hatching and naupliar longevity: *e.g.* Clegg (1962; 1967) observed decreased cyst viability with

increased storage time in air and at room temperature, with only 0.8 % of cysts hatching after 15 years and no remaining viable cysts after 28 years. Also Vanhaecke and Sorgeloos (1982) reported that harvesting, cyst drying techniques as well as storage conditions may significantly affect cyst quality, with inappropriate handlings resulting in a substantial decrease in hatchability. This is in line with the current results demonstrating that hatching % decreased significantly with time of storage (LMM; Table 9.1. and Fig. 9.1 in appendix C).

The individual energy content in both strains, being maximal in the control cysts, gradually declined as the number of H/D cycles and duration of hydration increased, with a loss of energy content more than three (3.5 – 3.8) times higher after 4 h of hydration compared to 2 h. Cysts lost more energy when being hydrated continuously for a certain period compared to being hydrated for twice half that period (4 h hydration (A2) produced more energy loss than twice 2 h (A11)). Double or triple H/D cycles, both for 2 and 4 h hydration did not result in a double or triple energy loss. After the first cycle of hydration, a number of metabolic mechanisms are initiated, which might not be repeated during the following cycles. So cyst development can be interrupted and each successive H/D cycle would bring further development, lowering the energy content, but not in a linear fashion. Clegg (1976) indicates that *Artemia* cysts initiate the utilization of the carbohydrate trehalose, through conversion into glycogen as a source of energy, only when hydration is taking place. This glycogen is consumed during the development of the cysts (Muramatsu, 1960; Clegg, 1964). Our results also showed a very strong positive correlation in both strains between hatching percentage after different storage periods, and individual energy content of cysts (Fig. 3.2. A and 3.2. B). A similar positive correlation was found between survival and energy content of starved nauplii (Fig. 3.3 A and 3.3 B). During the first stage, the nauplii do not feed and instead use the yolk from their cyst as energy (Benijts et al., 1976). However, the

linear mixed model failed to demonstrate a significant interactive effect ($P > 0.05$) between H/D treatment and storage time on cyst hatching, and between H/D treatment and starvation period on survival of nauplii (Table 9.1 and 9.2 in appendix C). This suggests that the effect of H/D treatment on hatching of stored cysts is not influenced by storage time (Table 9.2 and Fig. 9.2 in appendix C), and that the effect of H/D treatment on survival of nauplii is not influenced by the duration of starvation (Table and Fig. 9.2 in appendix C). The relationship between the energy content of cysts exposed to H/D cycles and the energy content and survival of the emerged nauplii, is important from a practical point of view. Fish or crustacean larvae may be fed with nutritionally poor live feed when *Artemia* cysts of unknown history and quality are used for hatching.

The total FAME level (µg $cyst^{-1}$ DW) of the A222 cysts sample (which had gone through the most extreme H/D treatment) was lower than in the control with a decrease of 10 % in both strains. For the individual control nauplii, the decrease was 30 – 40 % compared to the individual control cysts. This suggests that fatty acids may be used as energy resource during the H/D process of cysts and also further on in the process towards emergence of nauplii. On the other hand, the decrease in the A222 sample (mg g^{-1} DW) compared to the control nauplii was lower than when comparing A222 with control cysts, with a decrease of 1.7 % for GSL and 4.6 % for VC. The effect of the H/D cycles on the fatty acid content varied among the individual fatty acids or groups of fatty acids or when considering cysts versus nauplii. The net breakdown per cyst of some HUFAs (especially ARA) during the H/D process is of a comparable level as the loss in energy content. This suggests that fatty acids breakdown may be used as a source of energy for development in *Artemia* cysts along with other nutritional compounds. The emerged *Artemia* nauplii of the A222 sample showed little decrease in the total FAME (expressed as mg g^{-1} DW) as compared to the control nauplii, (see above), whereas they showed a decrease in energy content of 10.34 % for GSL and 8.13 %

for VC, and this may be related to the use of carbohydrates as energy reserve. According to Morris (1999) glucose delivers fast energy in the form of ATP via the process of glycolysis and oxidative phosphorylation and is the major circulating carbohydrate in crustacean larvae; carbohydrates are used before lipids and proteins as the preferred fuel for metabolic processes (Garret and Grisham, 1995). In our test *Artemia* energy content was declining with about 16 %, whereas lipid content was decreasing with about 10 %; as these values are based on a single measurement and are thus not statistically supported, it is difficult to say if lipid content decreases at a slower pace than overall energy content.

Just like HUFAs, vitamin C levels in live food such as *Artemia* are of nutritional importance (Merchie et al., 1997b); therefore, we wanted to assess if these are affected by successive H/D cycles. The loss amounted in both strains up to 60 – 70 % for the A222 cysts sample (µg $cyst^{-1}$ DW) in GSL and VC, respectively and about 10 % in A222 nauplii (mg g^{-1} DW) in both strains as compared to the control. The amount of vitamin C, more specifically ascorbic acid (AA), liberated in freshly hatched nauplii, reflects the ascorbic acid 2-sulfate (AAS) reserve present in the cysts, which is converted to free AA during completion of the embryonic development into nauplii (Golub and Finamore, 1972; Dabrowski, 1991; Nelis et al., 1994; Merchie et al., 1995a). However, this low level of vitamin C is much lower than the requirement of AA for optimal growth and survival of aquaculture species. For production of fish and shrimp juveniles, a minimum level to secure an optimal performance and enhance the resistance to diseases and stress conditions is 1 – 5 mg AA g^{-1} DW diet for fish (Agrawal et al., 1978; Ishibashi et al., 1992) and about 1.5 – 2 mg AA g^{-1} DW diet are suggested for shrimp (Kontara et al., 1997). Larval fish and shellfish, on the other hand, displaying a relatively faster growth and metabolism than juveniles and adults, might need higher dietary AA levels to sustain optimal growth and physiological conditions (Dabrowski,

1990); the levels found in our A222 decapsulated cysts and instar I nauplii (in the range 111 and 724 µg g^{-1} DW) are thus probably too low to satisfy these needs.

8.1.2. Effect of successive H/D cycles on the stress response of cysts and of the emerged nauplii

Several molecular compounds have been suggested as indicators of stress in animals. Among those components are the stress proteins or heat shock proteins (Hsps) that are implicated in embryo survival under life-threatening conditions (MacRae, 2010; Warner et al., 2010). Among these Hsps, mainly the 70 kilodalton Hsp (Hsp70) has been considered as the most prominent one being synthesized in response to stress. Therefore, in a subsequent step (Chapter 4) the effect of H/D cycles on the induction of Hsp70 and on the functional stress response (*i.e.* resistance towards abiotic and pathogenic biotic factors) of cysts and their emerged nauplii was evaluated in gnotobiotic conditions.

Quantification using a ChemiDoc MP Imaging System (BioRad, Belgium) of the SDS-PAGE gel revealed that in GSL H/D cycles induced Hsp70 production in the *Artemia* cysts in a manner dependent on the number of H/D cycles and the duration of the hydration phase. In the GSL cysts, H/D treatment markedly increased the Hsp70 level in all the treated samples compared to the control (Fig. 4.1 A and 4.1 B, cysts), but the opposite was the case for the VC cysts. The high level of Hsp70 produced in the GSL treated cysts is in agreement with the finding that when such cells are stressed by abiotic and biotic insults, there is up-regulation of the inducible form of Hsps (*i.e.* Hsp70), which can be detected in the cells at concentrations two or three times those of their constitutive counterpart (Pockley, 2003; Roberts et al., 2010). The reduction in the level of Hsp 70 (which could be both inducible and constitutive) in the treated VC cysts compared to the control

might mirror the higher standing/basal level of this protein in this strain, reflecting the adaptation to thermal stress in its natural habitat that might suffice when exposed to additional stress. The differences between the two strains in the induction pattern of Hsp70 in response to H/D treatment could thus be associated to the environmental conditions in the two different geographical locations, from where these two strains originated. According to Clegg et al. (2000), the cysts produced in Vietnam, are much more resistant to high temperatures than cysts produced in Great Salt Lake. Maximum water temperatures in GSL during summer and spring very rarely exceed 24°C while the Vietnamese *A. franciscana* strain experiences daily water temperatures near to 38 °C for most of the growing season, suggesting that VC cysts have become adapted to higher temperatures (Clegg et al., 2001 and references therein). VC *Artemia* might also experience higher daily variation in temperature as they are produced in shallow small scale ponds, which is a trigger for Hsp production. In addition to chapter 4, new statistical analysis (a two-way ANOVA model) was run to determine the overall effect of each single challenge separately (thermotolerance and *Vibrio* challenge assay). This analysis showed that in the thermal shock assay interaction between H/D treatment and strains was highly significant (P-values < 0.0001) (Table. 10.1), whereas this was not the case for the *Vibrio* challenge assay (Table. 10.1, $P > 0.05$). This indicates that the two strains responded differently when challenged with thermal shock, but similarly when *Vibrio* challenged. When challenged with thermal shock or *Vibrio* only the nauplii emerged from the cysts exposed to a mild H/D treatment had a survival similar (in case of VC) or significantly higher (for GSL) than in the control. However, increasing the number of H/D cycles or prolonging the hydration period significantly reduced the survival of the thermally stressed and *Vibrio* challenged nauplii (Fig.10.1 and 10.2). This indicates that starved nauplii with low energy reserves become weak and less protected against different types of stressors. When nauplii are challenged with

pathogenic bacteria, the non-specific innate immune system is activated which also needs energy. Therefore, when there is a low energy reserve due to previous H/D cycles, the nauplii may show more mortality when exposed to the pathogen. A mild H/D treatment may not reduce (in case of VC) and even improve (in case of GSL) stress resistance in *Artemia* nauplii, whereas a more pronounced H/D treatment does reduce stress resistance. The cross-protective effect observed in the case of the thermally stressed A1 GSL nauplii, after a mild H/D treatment, might be closely linked to the expression of Hsp70, agreeing with the results of Sung et al. (2007) who indicated the role for Hsp70 in cross-protection of *Artemia* larvae.

8.1.3. Effect of different light parameters and exposure to chemicals on hatching of *Artemia* cysts

Based on the results obtained in the second part of our thesis (Chapters 6 and 7), the hatching percentages of the different strains obtained after any duration of light exposure were significantly higher ($P < 0.05$) than under continuous darkness. Among the three light colours tested in this study, white light (a combination of different wavelengths in the visible spectrum) showed the highest positive effect on hatching percentage. Blue light was also suitable for triggering hatching, as opposed to red light which promoted hatching to a lower degree. This may be due to the fact that the haematin pigments of *Artemia* cysts may absorb light of short wavelength (*i.e.* blue light) and have little sensitivity to light of longer wavelength (*i.e.* red light). Results by Van Der Linden et al. (1986) indicated that a shorter exposure period to light with higher energy (*i.e.* 450 nm) gives a better hatching response than a longer exposure time with lower energy. In addition, our results suggest that a relatively short exposure of 2 – 6 h to white light is most effective in triggering hatching when supplied beyond complete hydration, and the hatching % obtained in these conditions was not significantly different compared to the continuous 24 h exposure. Further

extension of light exposure continuously over a period of 48 h resulted in only marginal increase in hatching %. This confirms the role of a minimum duration of light exposure demonstrated by Van Der Linden et al. (1985), needed to obtain maximal hatching.

Besides the duration of light exposure, also the timing of light exposure can be a determining factor for the cyst hatching process. In our observation, the exposure to white light had the highest efficiency when supplied during the 4th hour of incubation (hence well beyond complete hydration) and suboptimal when supplied early (1st h, while hydration is still ongoing) or later (8th h and especially the 13th h).

These results suggest that light is maximally effective after complete hydration of the cyst, as also shown for different *Artemia franciscana* strains from Utah and California (Sorgeloos, 1973), *Artemia urmiana* (Asil et al., 2012) and freshwater fairy shrimp (Pinceel et al., 2013). This period corresponds with the early hours of the onset of metabolism (Morris, 1971; Lavens and Sorgeloos, 1987). Within the context of a fish or shellfish hatchery, where hatching of *Artemia* cysts is a daily routine and the hatching observed during the first 24 h of the incubation for hatching is generally of practical relevance, a short illumination during the first 6 h of the incubation for hatching should thus be provided. This could be an adequate method for saving costs of power for hatching cysts in the larger hatcheries of shrimp and marine fish larvae.

In addition, variations were also observed between samples in our study; these might be attributed to strain differences in shell characteristics, such as chorion thickness, but it is not clear if the thin chorion of VC (half the thickness of the other two strains) is linked to the relatively high hatching of the VC strain in darkness. Van Der Linden et al. (1986) reported that a higher hatching percentage was observed in decapsulated cysts than in non-decapsulated cysts and assigned a light screening role to the haematin in the cyst shell. Although we did not determine this in our study,

it has been observed that photosensitivity may also be related to haem pigment (haematin) intensities in the outer cyst shell and depending on pigmentation, light may be reflected or absorbed at the surface (Gilchrist and Green, 1960; Van Der Linden et al., 1986). Variation in pigmentation could be an innate trait but could also result from environmental influences. Several studies suggested that *Artemia* cysts are susceptible to light triggering, mediated through a photoreceptor (haem pigments) when hydrated under aerobic conditions (Sorgeloos and Persoone, 1975) or through the involvement of haematin in the cyst shell combined with a haem protein photoreceptor in the gastrula to regulate hatching (Gilchrist and Green, 1960; Van Der Linden et al., 1986). All these factors may contribute to differences in transmission of light through the egg shell across different strains and batches of cysts.

Additionally, light exposure may have a different effect on diapausing versus on post-diapausing quiescent embryos, contributing to the differences observed between the different samples. Our limited set of strains did not cover the entire diapause/quiescence biodiversity within the genus *Artemia* and did not allow unravelling the possible role of the factors mentioned above.

Aside from the contribution of light, we also showed in our study that exposure to chemicals (H_2O_2 or NO) had a considerable enhancing effect on the hatching of diapausing and post-diapausing (quiescent) cysts, with the effect of H_2O_2 being more prominent. This observation is in agreement with the results of previous reports showing that the two chemicals influence physiological and developmental processes in many organisms (Stone and Yang, 2006; Bright et al., 2006; Giorgio et al., 2007; Zhang et al., 2007; Covarrubias et al., 2008; Zhao and Shi, 2009), including the germination of seeds, biological structures that share characteristics with *Artemia* cysts (for review see Robbins et al., 2010 and references therein). The enhancing effects of these two chemicals can be linked to their reactive mechanisms, for example H_2O_2 can be either activating or inhibiting

regulatory, metabolic and structural proteins of the cysts (Robbins et al., 2010), leading to a condition in which the cysts sense the environment as being favourable for survival and thus terminate diapause and proceed into the development process.

Furthermore, the results demonstrated that in combination with white or blue light, chemicals exposure (H_2O_2 or NO) can exert a strong effect on *Artemia* cysts hatching percentage beyond that obtained by the separate treatments. A synergistic effect was obtained between exposure to H_2O_2 and exposure to blue or white light for the *Artemia* sample in diapause state (*i.e.* TK strain). In the VC sample on the other hand, which was predominantly quiescent, only an additive effect was observed when H_2O_2 was applied together with blue or white light. The BY sample showed an effect that we may call compensatory, in the sense that exposure to both H_2O_2 and blue or white light only showed limited hatching increase as compared to exposure to each factor separately. This difference in joined effect of light and chemical between the three strains might be related to the diapause or non-diapause state of the cysts (Van Stappen et al., 1998). In cysts out of diapause (the VC sample), chemicals have no effect and only light is needed for hatching. In cysts in diapause (*e.g.* TK; demonstrating low hatching in light in optimal hatching conditions) chemicals may break diapause and light might terminate quiescence. The fact that there is a synergistic effect suggests that in biochemical terms diapause breakage and quiescence termination can be coupled, reinforcing each other. The details of such biochemical coupling remain elusive. BY cysts show an effect that we may name a compensatory pattern. This might not be the consequence of a different biochemical process, rather it could be the result of the fact that this sample contains a mixture of cysts, partly in diapause and partly out of diapause. To further unravel the diapause/quiescence puzzle, it would definitely be worthwhile to expose cysts sequentially to chemicals and light (with intermediate hatching verification; as also done by Robbins et al., 2010)

rather than simultaneously as performed in this study. Finally, the different hatching patterns shown by VC, BY and TK as influenced by light and H_2O_2 could become the basis for the characterization of cysts relative to their diapause state. Obviously that should be validated with a larger set of samples.

Overall, standardization of the production conditions that cysts and nauplii encounter, from the moment that they are released from the female until the point that they are used as live food, can lead to avoiding a potential *Artemia* production bottleneck and would sustain growth in aquaculture. The availability of high-quality *Artemia* cysts and nauplii as live food is a prerequisite for sustainable development and increased productivity of larval aquaculture.

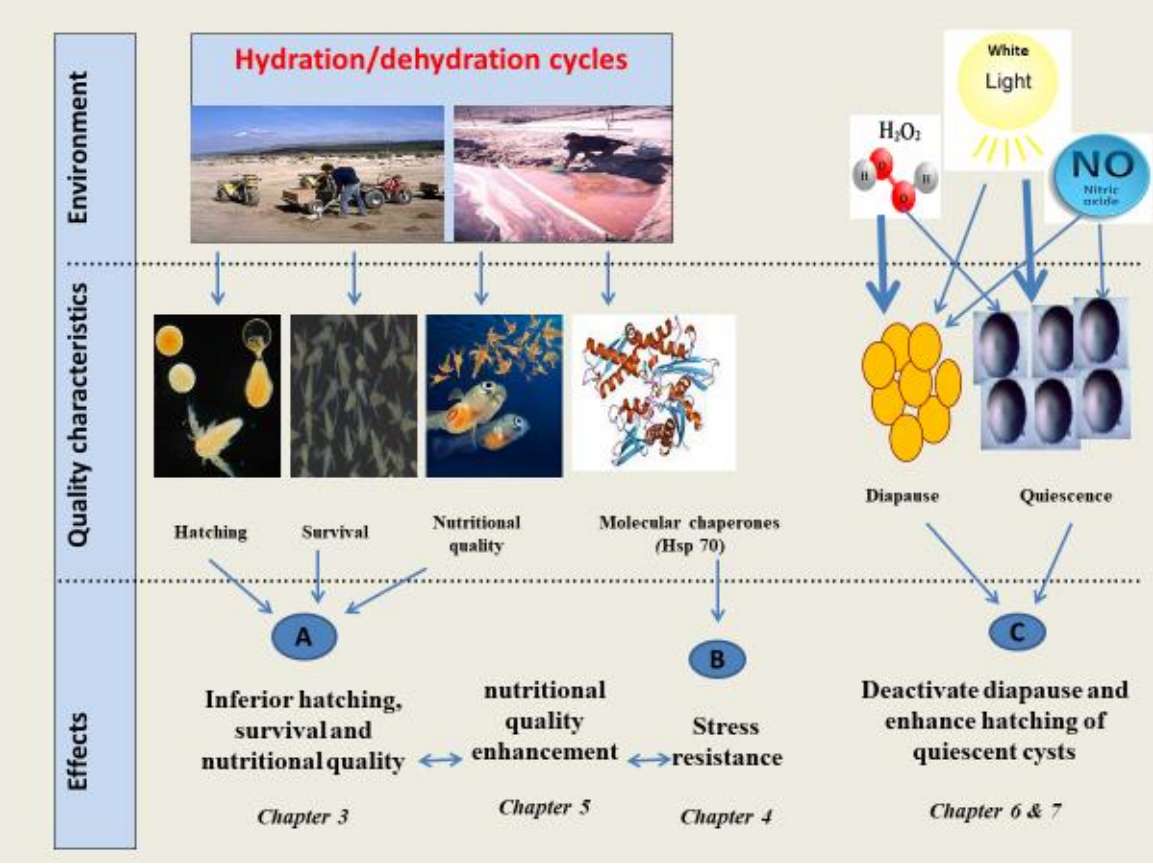

Figure 8.1: Diagram summarizing (A) the effects of H/D cycles as environmental factors on hatching, survival, nutritional quality, Hsp 70 levels of cysts and nauplii, and (B) how the status can be enhanced by adequate feeding; (C) effects of light and chemicals on diapause deactivation and enhancement of hatching of quiescent cysts. The thickest arrows indicate the more important effect of light and chemicals on diapausing and quiescent cysts.

8.2. General conclusions

Figure 8.1 provides a schematic overview of the research performed within the framework of this PhD research and of its most important conclusions. The following conclusions can be drawn:

- Repeated H/D cycles result in significantly decreased cyst hatching, reduced starved naupliar longevity and individual energy content, loss in vitamin C and fatty acid content (**chapter 3**). Moreover, a close correlation between these parameters was observed as a function of progressive H/D treatments.

- The results obtained in **chapter 4** and **5** on the performance of *Artemia* in a gnotobiotic culture system illustrated that *Artemia* nauplii emerged from cysts exposed to a limited H/D treatment may show enhanced protection against stressors, and this protection may be modulated by nutritional factors. More pronounced H/D treatments may result in the opposite effect. Moreover, the results reported in this study suggest a different stress response strategy of *Artemia* originating from different habitats.

- Light has a high effect in stimulating hatching, but differently when three different colours are used. Exposure to H_2O_2 or NO also enhanced hatching percentage, but the effect of H_2O_2 was more prominent. In combination with light conditions, exposure to chemicals (H_2O_2 or NO) (**chapter 6 and 7**) exerted a higher effect on *Artemia* cysts hatching beyond that obtained by the separate treatments: when using blue or white light with H_2O_2 a synergistic effect was obtained for the *Artemia* sample in diapause (*i.e.* TK strain), while for the quiescent VC sample an additive effect was observed.

8.3. Future perspectives

- In order to augment our knowledge on the effects of H/D cycles on the cyst metabolism, it may be worthwhile to study how the level of other nutritional compounds such as proteins, free amino acids, carbohydrates, evolves as a function of H/D cycles. Studying the effects of others factors such as UV radiation, oxidation, temperature, salinity etc. on cyst and nauplius quality could provide valuable additional information.

- For more efficient use of the natural *Artemia* resources, better management and further rationalization of harvesting, processing and storage procedures, handling and manipulation could be applied. As cysts floating on the water surface are less exposed to H/D cycles than those accumulating on the shore, harvesting from the shore should be avoided.

- The link between cyst quality and quality of the emerged nauplii could be used as an indicator to predict nauplii quality based on cyst composition.

- The relationship between H/D treatments and stress protection could be further unraveled by further studying the role of Hsp70 and other stress proteins, which could then be used as an indicator of cyst quality. Moreover, also the levels of other molecular compounds involved in stress protection could be studied in the hydration/dehydration process, such as trehalose and the small heat shock proteins (*i.e.* p26 and artemin). Moreover, studies could be conducted on other feed components, including probiotics, improving the performance of nutritionally deficient *Artemia*, used as live food in aquaculture.

- Future studies should address how factors such as timing and duration of light exposure interfere with the hatching metabolism. Also the link between the effects of light exposure and treatment with different chemicals, and how they interfere with the physiological and developmental

processes of the hatching metabolism and with diapause deactivation, should be further investigated.

- From a practical point of view, in many hatcheries light conditions are not optimized during hatching; maybe new technologies could be developed to treat cysts during processing, so that they can hatch in full darkness or when being exposed to conditions of limited light energy. It would be worthwhile to study if the light stimulus, received by the embryo, can be stored by the organism for later hatching.

- Finally, employing advanced tools such as the information made available through the annotated *Artemia* genome will allow obtaining a better in-depth insight into the mechanisms behind the observed effects of various factors on the hatching process. Organisms respond to biotic or abiotic environmental stressors with a battery of adaptive phenotypes (*e.g.* increased resistance, protein production) which can be assessed by gene expression measurements.

Appendix A

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A

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Appendix B

Summary

In view of the urgent necessity to improve the quantity and the quality of the brine shrimp *Artemia* as a live food in finfish and shellfish hatchery operations, there is a strong need to improve our basic knowledge on the impacts of environmental stresses and manipulations during processing on *Artemia* cysts and their emerging nauplii. A high variety of cyst products is offered on the market, but knowledge about the effects of these factors which could lead to variation of the product quality is still poor. This includes the effect on breaking the *Artemia* cyst diapause, a complex process that requires a lot more understanding. Therefore, in the present study, the effects of different environmental factors were investigated on cyst and naupliar quality and on cyst hatching.

Firstly, the effect of abiotic conditions (*i.e.* hydration/dehydration (H/D) cycles) on the life processes in *Artemia franciscana* cysts from Great Salt Lake, USA and Vinh Chau, Vietnam, and in the emerged nauplii was assessed. Results evidenced that repeated H/D cycles resulted in significantly decreased cyst hatching, reduced longevity of starved nauplii, lower cyst energy content, loss in vitamin C and fatty acid content. Moreover, a close correlation between these parameters was observed as a function of progressive H/D treatments. This effect aggravated as the number of H/D cycles increased from one to three, and as the hydration period, preceding dehydration, was lengthened from 2 to 4 h. The results also show that cyst metabolism, as initiated after hydration, is to a certain degree interruptable and that cysts can be converted from a hydrated, metabolically active mass of cells into a dehydrated, ametabolic state.

Subsequently, this work also presents a study assessing the effects of H/D cycles on the resistance towards abiotic (thermal) and biotic (pathogenic bacteria) stress using the emerged gnotobiotic

Artemia nauplii as model organism. Results showed that successive H/D cycles appeared to have a deleterious effect on resistance against these stressors, whereas a limited exposure resulted in the opposite effect and may lead to the induction of thermotolerance in nauplii and an up-regulation of Hsp70 in cysts. The results reported in this study thus augment our current understanding of stress responses in *Artemia*. Furthermore, suitable feeding was found to have an important role in protecting *Artemia* nauplii, exposed to these stressors, when emerged from cysts exposed to a mild H/D treatment; following more extreme H/D treatments on the other hand, the feeding proved less instrumental in protecting the nauplii.

Secondly, the effect of illumination on the hatching of cysts of different *Artemia* strains was investigated. The results gave an indication that the optimal timing and duration of light exposure during the hatching process plays an important role. A relatively short exposure to white light can maximally trigger the hatching process. Blue light (of short wavelength) was also suitable for triggering hatching, as opposed to red light (of longer wavelength) which promoted hatching at lower amounts. Besides the duration of light exposure, also the timing of light exposure can be a determining factor for the cyst hatching process. Light had the highest efficiency when supplied during the initial hours of incubation, beyond complete hydration. The existence of differences in the effect on the hatching process observed between samples may be linked to factors such as chorion thickness or concentration of haem pigments in the cyst shell, which may affect the extent to which light can penetrate through the shell to the embryos, which is necessary for development. In addition, light exposure may have a different effect on diapausing versus on post-diapausing quiescent embryos, contributing to the differences observed between the different samples. Our limited set of strains did not cover the entire biodiversity within the genus *Artemia* and did not allow unraveling the possible role of the factors mentioned above.

Finally, the effect of chemicals (H_2O_2 and NO) separately or in combination with light was investigated to find out whether there is any interaction between the effect of light and the effect of these chemicals on the *Artemia* hatching process. The results demonstrated that H_2O_2 or NO also had considerable enhancing effects on hatching percentage, but the effect of H_2O_2 was more prominent. In combination with light, exposure to chemicals (H_2O_2 or NO) exerted a higher effect on *Artemia* cysts hatching beyond that obtained by the separate treatments. For cysts out of diapause (the Vinh Chau sample, Vietnam), chemicals had no effect and only light was needed for hatching. In cysts in diapause (the sample from Tuz Lake, Kazakhstan) chemicals may break diapause and light might terminate quiescence. The fact that there was a synergistic effect between exposure to H_2O_2 and exposure to blue or white light in this sample, suggests that in biochemical terms diapause breakage and quiescence termination can be coupled, reinforcing each other. Finally, combining chemicals with blue or white light in cysts from Bolshoye Yarovoye, Russia, showed a compensatory effect between both factors. This might not be the consequence of a different biochemical process, rather it could be the result of the fact that this sample contained a mixture of cysts, partly in diapause and partly out of diapause. These results may be applicable for cysts locked in the diapause state, which is sometimes a bottleneck in experimental studies and applications. They may help in improving protocols that result in increased hatching and more predictable diapause termination. For a more efficient use of the natural *Artemia* resources, better management and further rationalization of harvesting, processing and storage procedures, handling and manipulation should be applied.

Appendix C

Samenvatting

Gezien de dringende noodzaak om de kwantiteit en kwaliteit te verbeteren van het pekelkreeftje *Artemia* als levend voedsel in broedhuizen van vissen en schelpdieren, is het hoogst noodzakelijk om onze fundamentele kennis te vergroten over de invloed op *Artemia*-cysten en de ontluikende nauplii, uitgeoefend door omgevingsstressoren en behandelingen tijdens de verwerking. Een grote variëteit aan cystproducten wordt aangeboden op de markt, maar de kennis is beperkt van de effecten van deze factoren, die kunnen leiden tot een variabele productkwaliteit. Dit omvat ook het effect op het beëindigen van de diapause in *Artemia*-cysten, een complex process dat een beter begrip behoeft. Daarom werden in deze studie de effecten van verschillende omgevingsfactoren onderzocht op de kwaliteit van cysten en van nauplii, en op cystontluiking.

Eerst werd het effect onderzocht van abiotische omstandigheden (i.e. hydratatie/dehydratatie (H/D) cycli) op de levensprocessen in cysten en ontloken nauplii van *Artemia franciscana* afkomstig van Great Salt Lake, USA, en van Vinh Chau, Vietnam. De resultaten toonden aan dat herhaalde H/D cycli resulteerden in significant verminderde cystontluiking, verminderde levensduur bij nauplii die geen voedsel kregen, verminderde energie-inhoud van de cyst en verlies aan vitamine C en vetzuren. Bovendien werd een nauwe correlatie vastgesteld tussen deze parameters in functie van toenemende H/D behandeling. Dit effect werd groter naarmate het aantal cycli toenam van één tot drie, en naarmate de hydratatieperiode voorafgaand aan de dehydratatie, verlengd werd van 2 tot 4 u. De resultaten toonden ook aan dat het cystmetabolisme, dat geïnitieerd wordt na hydratatie, tot op zekere hoogte onderbroken kan worden, en dat cysten kunnen omgezet worden van een gehydrateerde, metabolisch actieve celmassa naar een gedehydrateerde ametabolische status.

Vervolgens presenteert dit werk ook een studie die peilt naar de effecten van H/D cycli op de weerstand tegen abiotische (thermische) en biotische (pathogene bacteriën) stress, waarbij gebruik gemaakt werd van gnotobiotische *Artemia*-nauplii als modelorganisme. De resultaten toonden aan dat opeenvolgende H/D cycli blijkbaar een negatief effect hadden op de weerstand tegen deze stressoren, terwijl een beperkte blootstelling een omgekeerd effect had en kon leiden tot de inductie van thermotolerantie bij nauplii en upregulatie van Hsp70 in cysten. De resultaten gerapporteerd in deze studie vergroten dus ons huidig begrip van stress-respons in *Artemia*. Verder vonden we dat een geschikt diet een belangrijke rol speelde bij de bescherming van *Artemia*-nauplii, blootgesteld aan deze stressoren, wanneer deze nauplii ontloken uit cysten die een matige H/D behandeling hadden ondergaan. Na meer extreme H/D behandeling echter, was het diet minder van nut om de nauplii te beschermen.

Ten tweede werd het effect van belichting op het ontluiken van cysten van verschillende *Artemia*-rassen onderzocht. De resultaten gaven een indicatie dat optimale timing en duur van blootstelling aan licht een belangrijke rol speelt tijdens het ontluikingsproces. Een relatief korte blootstelling aan wit licht kan het ontluikingsproces maximaal triggeren. Blauw licht (van korte golflengte) was ook geschikt om de ontluiking te triggeren, in tegenstelling tot rood licht (van langere golflengte), dat slechts tot beperkte ontluiking leidde. Naast de duur van blootstelling kan ook de timing van blootstelling een bepalende factor zijn in het ontluikingsproces. Licht was maximaal efficiënt wanneer het ingezet werd tijdens de initiële uren van de incubatie, nadat volledige hydratatie van de cysten was bereikt. De vastgestelde verschillen in effect op ontluiking tussen de stalen kunnen verband houden met factoren zoals choriondikte of concentratie aan haempigmenten in de cystschaal, die kunnen bepalen in welke mate licht door de schaal kan doordringen tot bij de embryos, wat noodzakelijk is voor ontwikkeling. Bovendien kan licht een verschillend effect

hebben op cysten in diapauze versus quiescente embryos post-diapauze, hetgeen kan bijdragen tot de verschillen vastgesteld tussen de stalen. Onze beperkte set aan stalen dekte niet de volledige biodiversiteit binnen het genus *Artemia*, en liet niet toe om de mogelijke rol van hoger genoemde factoren te ontrafelen.

Tenslotte onderzochten we het effect van chemicaliën (H_2O_2 en NO), apart en in combinatie met licht, om te achterhalen of er een interactie is tussen het effect van licht en van deze chemicaliën op het ontluikingsproces in *Artemia*. De resultaten toonden aan dat H_2O_2 of NO ook het ontluikingspercentage aanzienlijk verhoogden, maar het effect van H_2O_2 was hoger. Gecombineerd met licht, had blootstelling aan chemicaliën (H_2O_2 of NO) een groter effect op ontluiking vergeleken met het effect verkregen met de aparte behandelingen. Voor cysten uit diapauze (het staal van Vinh Chau, Vietnam), hadden chemicaliën geen effect en alleen licht was nodig voor ontluiking. Bij cysten uit diapauze (het staal van Tuz Lake, Kazakstan) kunnen chemicaliën de diapauze stopzetten en licht kan de quiescentie beëindigen. Het feit dat er een synergistisch effect was bij dit staal tussen blootstelling aan H_2O_2 en blootstelling aan blauw of wit licht, suggereert dat in biochemische termen de beëindiging van diapauze en van quiescentie kan gekoppeld worden, waarbij ze elkaar versterken. Tenslotte toonde de combinatie van chemicaliën met blauw of wit licht in cysten van Bolshoye Yarovoye, Rusland, een compensatorisch effect tussen beide factoren. Dit is mogelijk niet het gevolg van een verschillend biochemisch process, maar eerder van het feit dat dit staal bestond uit een mengsel van cysten, gedeeltelijk in en gedeeltelijk uit diapauze. Deze resultaten kunnen van toepassing zijn op cysten die in diapauze vergrendeld zitten, wat soms een bottleneck is bij experimentele studies en toepassingen. Ze kunnen helpen bij het verbeteren van protocols die leiden tot verbeterde ontluiking en tot meer voorspelbare beëindiging van diapauze. Voor een meer efficiënt gebruik

van de natuurlijke voorraden van *Artemia*, zijn een beter management en verdere rationalisatie nodig van procedures voor het oogsten, verwerken, opslaan en manipuleren van *Artemia*-cysten.

Appendix D

Updated statistical analysis of chapter 3

9.1. Statistical analysis:

To evaluate the effect of different H/D cycles on hatching and survival percentages for two strains of nauplii (GSL and VC) after different duration of storage, a linear mixed effect model was used in SAS (SAS version 9.4, SAS Institute, Cary, NC) (Verbeke, G. and Molenberghs, G. 2000). Hatching and survival percentage data (the dependent variables) were normalized using the arcsin-square-root transformation prior to performing statistical analysis, while only non-transformed means are presented in the plot. The fixed effects (independent variables) used were time (different duration of storage) and treatments (different duration and number of H/D cycles). Interaction effects between different duration of storage and treatments (H/D cycles) were assessed for significance ($P < 0.05$). All pairwise treatment comparisons were done with a Tukey's post hoc adjustment method for multiple testing (Sherri, 2012).

9.2. Results

9.2.1. Hatching % of cysts

Table 9.1: Results of linear mixed model for GSL and VC strains showing effect of interaction between the different duration of storage and the H/D treatments on the hatching % of cysts.

Test of fixed effects								
Strain	**GSL**				**VC**			
Effect	Num. DF	Den. DF	F-Value	*P*-value	Num DF	Den DF	F-value	*P*-value
Treatment	6	14	445	<.0001	6	14	1226.8	<.0001
Time	2	28	8.9	0.0010	2	28	36.8	<.0001
Treatment*Time	12	28	0.61	0.8171	12	28	2.02	0.0613

Num. DF = Numerator degree of freedom. Den. DF = Denominator degree of freedom.
GSL = Great Salt Lake. VC = Vinh Chau. A1 = 2h hydration + 24 h dehydration (1 cycle). A2 = 4 h hydration + 24 h dehydration (1 cycle), A11 = 2 h hydration + 24 h dehydration (2 cycles). A22 = 4 h hydration + 24 h dehydration (2 cycles), A111 = 2 h hydration + 24 h dehydration (3 cycles). A222 = 4 h hydration + 24 h dehydration (3 cycles).

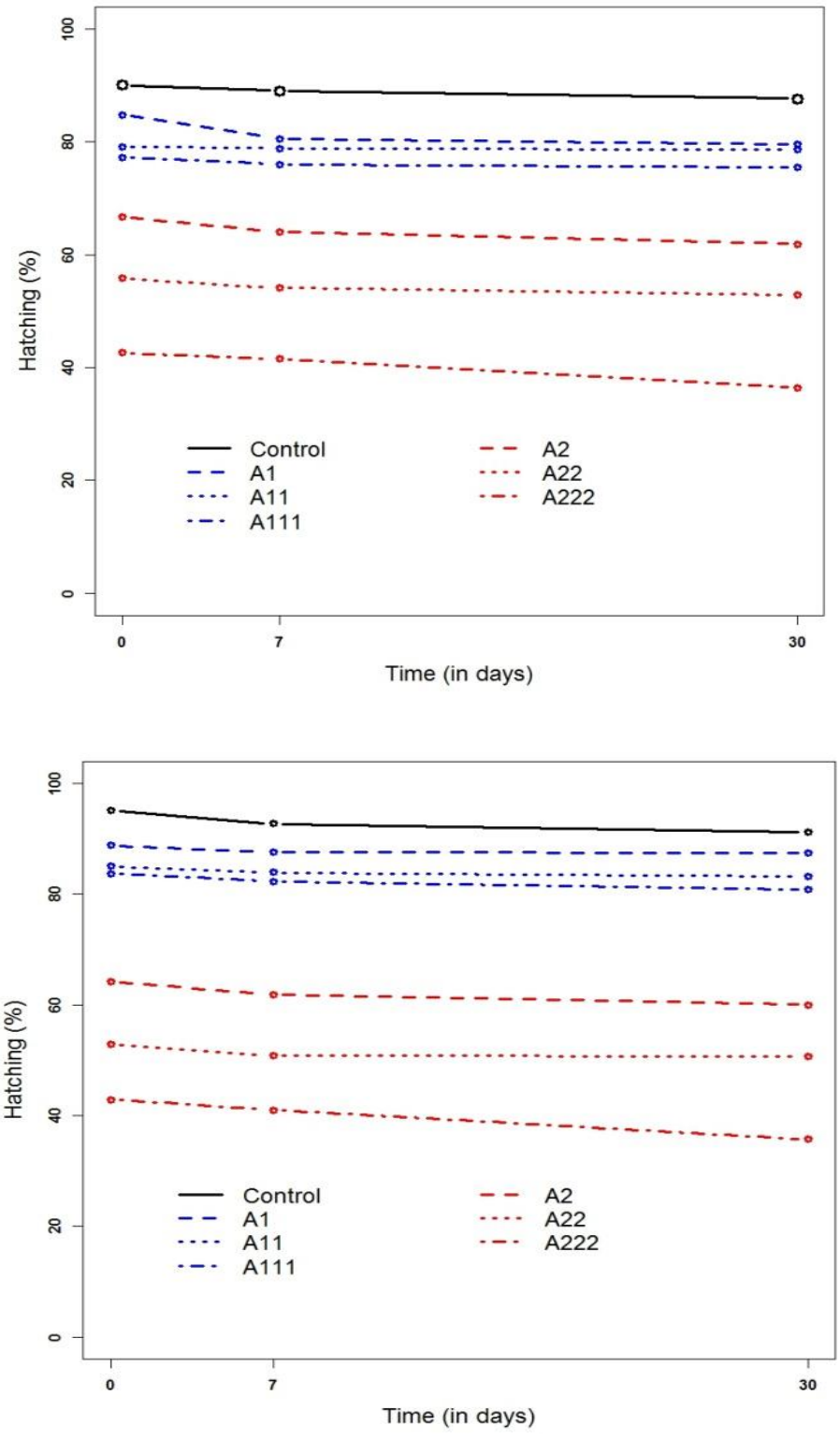


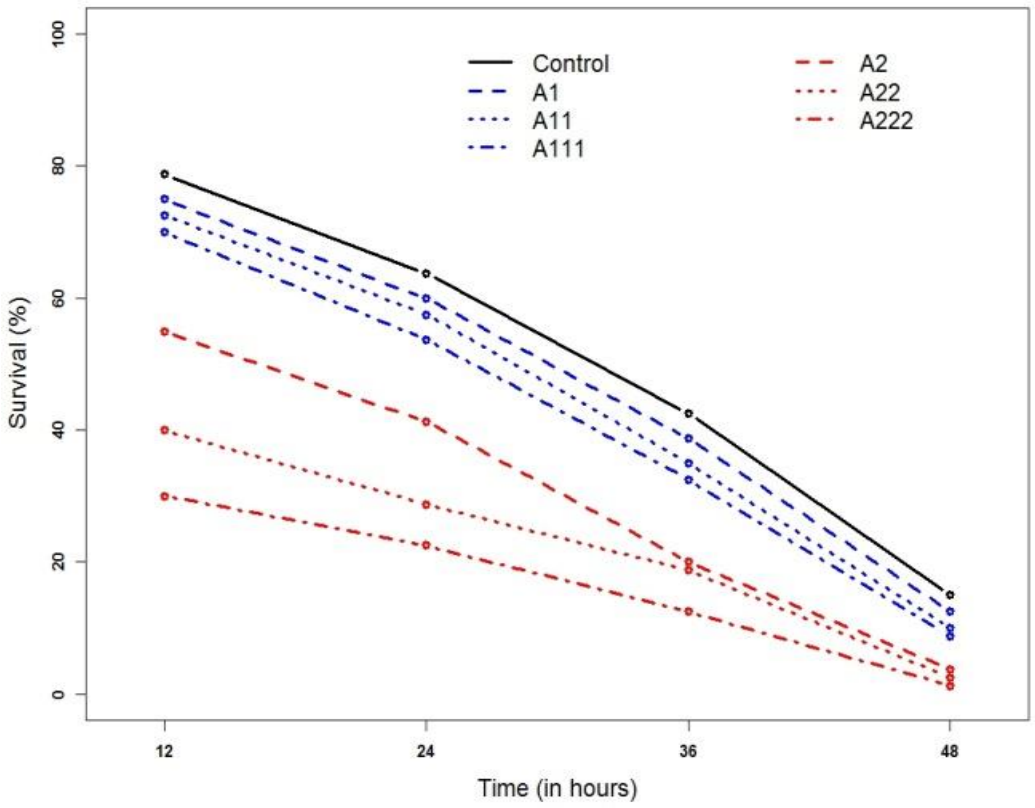
Figure 9.1: Line plot of hatching % of decapsulated GSL (top) and VC (below) cysts previously exposed to various H/D treatments (A1, A11, A111, A2, A22 and A222, respectively) after different storage periods (day 0, after 1 week, and 1 month, respectively). For abbreviations, see Table 9.1.

9.2.2. Survival of starved nauplii

Table 9.2: Results of linear mixed model for GSL and VC strains showing effect of interaction between the different starvation periods and the H/D treatments.

Test of fixed effects								
Strain	**GSL**				**VC**			
Effect	Num. DF	Den. DF	F-Value	*P*-value	Num. DF	Den. DF	F-value	*P*-value
Treatment	6	84	71.71	<.0001	6	84	52.63	<.0001
Time	3	84	401.86	<.0001	3	84	300.76	<.0001
Treatment*Time	18	84	0.97	0.4968	18	84	0.33	0.9951

For abbreviations, see Table 9.1.



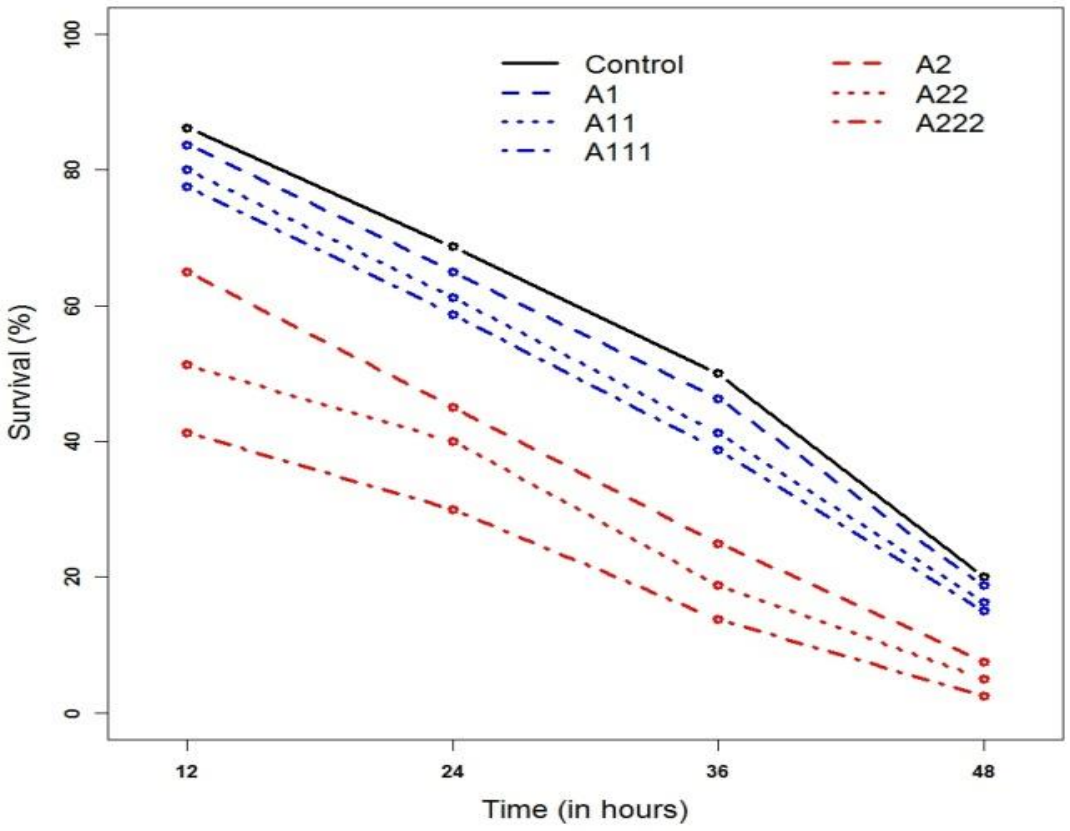


Figure 9.2: Line plot of survival % of the nauplii of GSL (top) and VC (below) cysts previously exposed to various H/D treatments (A1, A11, A111, A2, A22 and A222, respectively) after different starvation periods (12, 24, 36 and 48 h, respectively). For abbreviations, see Table 9.1.

Appendix E

Updated statistical analysis of chapter 4

10.1. Statistical analysis:

Survival % data were arcsin-square-root transformed to make them more normal and to enhance the suitability of the data to the frame work of the models (Warton and Hui, 2012). To determine the overall effect of each single challenge assay separately (thermal shock or pathogenic bacteria) on the survival percentage (as a dependent variable) of two strains of nauplii (VC and GSL), a two-way ANOVA model was fitted to the data using the SAS software (SAS version 9.4, SAS Institute, Cary, NC). The two independent variables (H/D treatments and strains) and their two-way interactions were evaluated for significance at the 5% significance level. Comparisons of differences in survival % between H/D treatments were done using the Tukey post hoc method which adjusts for multiplicity and reports adjusted *P*-values (Sherri, 2012). In addition, variations in survival percentage were explored using bar plots.

10.2. Results

10.2.1. *Thermotolerance of nauplii hatched from cysts exposed to successive hydration/dehydration treatments*

Table 10.1: Results of two-way ANOVA model showing interaction between strains and treatments (H/D cycles) for thermotolerance assay.

Tests of fixed effects				
Effect	Num. DF	Den. DF	F-Value	*P*-value
Strain	1	56	52.71	<.0001
Treatment	6	56	104.52	<.0001
Strain*Treatment	6	56	4.86	0.0005

For abbreviations, see Table 9.1.

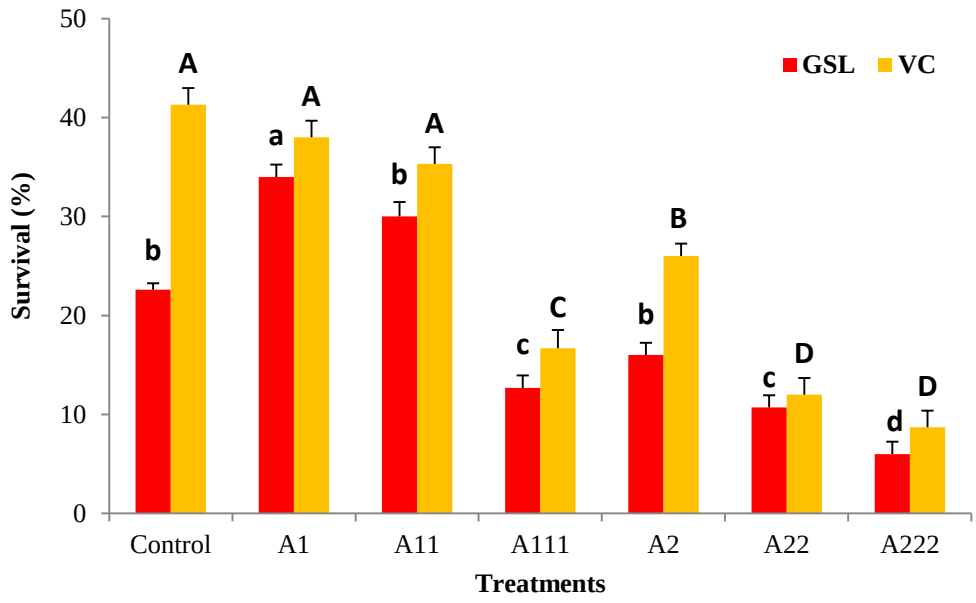


Figure.10.1: Survival (%) of *Artemia* nauplii after 36 h of challenge with thermal shock. The nauplii hatched under axenic conditions from GSL and VC cysts previously exposed to H/D treatments (A1, A11, A111, A2, A22 and A222, respectively). The challenge type was a lethal heat shock at 41°C for 20 min (n = 5). For abbreviations, see Table 9.1. For each treatment small and capital letters above bars indicate significant differences ($P < 0.05$) from the corresponding control.

10.2.2. *Resistance against V. campbellii of nauplii hatched from cysts exposed to successive hydration/dehydration treatment*

Table 10.2: Results of two-way ANOVA model showing interaction between strains and treatments (H/D cycles) for *Vibrio campbellii* challenge assay.

Tests of fixed effects				
Effect	Num. DF	Den. DF	F-Value	*P*-value
Strain	1	56	309.15	<.0001
Treatment	6	56	28.99	<.0001
Strain*Treatment	6	56	1.64	0.1522

For abbreviations, see Table 9.1.

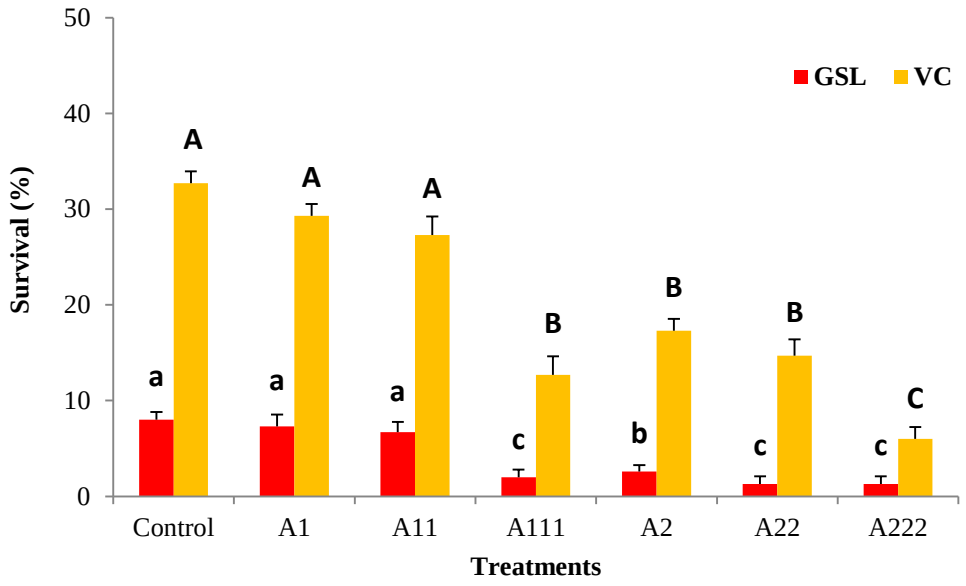
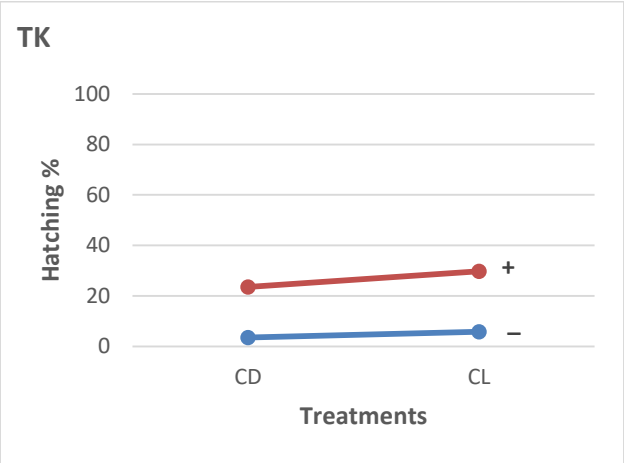
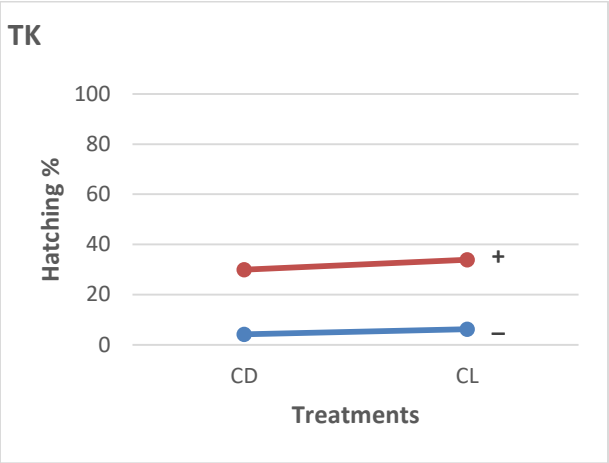


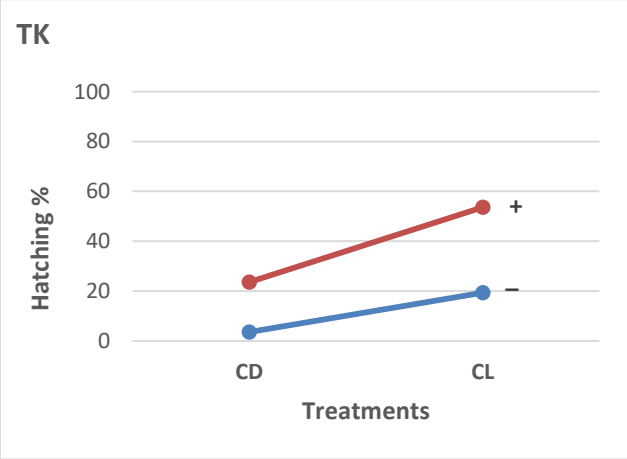
Figure. 10.2: Survival (%) of *Artemia* nauplii after 36 h of challenge with pathogenic bacteria. The nauplii hatched under axenic conditions from GSL and VC cysts previously exposed to H/D treatments (A1, A11, A111, A2, A22 and A222, respectively). The challenge type was with 10^7 cells/mL of *V. campbellii* (n = 5). For abbreviations, see Table 9.1. For each treatment small and capital letters above bars indicate significant differences ($P < 0.05$) from the corresponding control.

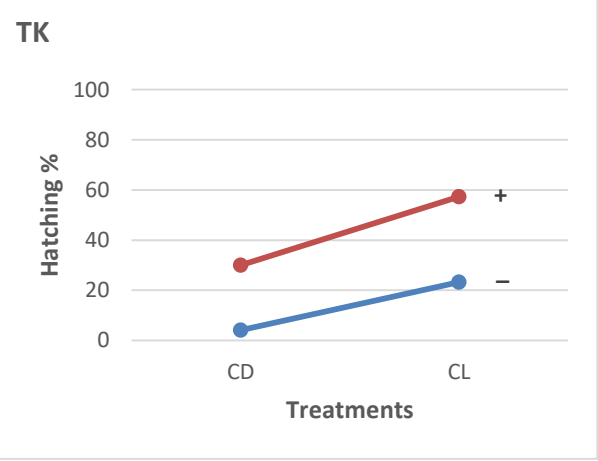
Appendix F

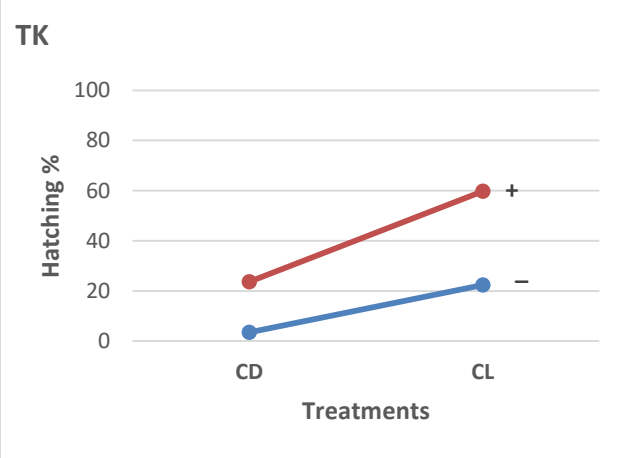
Additional data of chapter 7

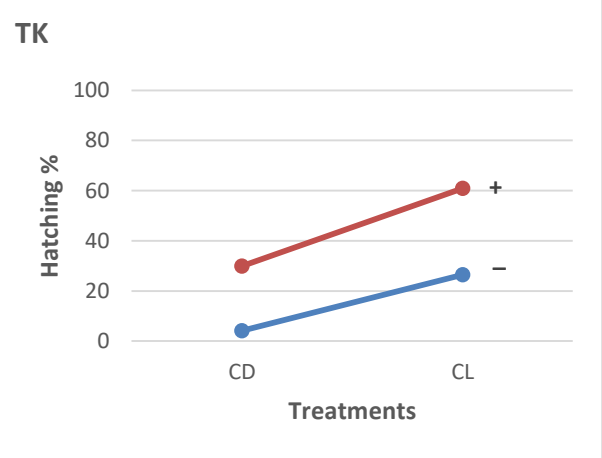


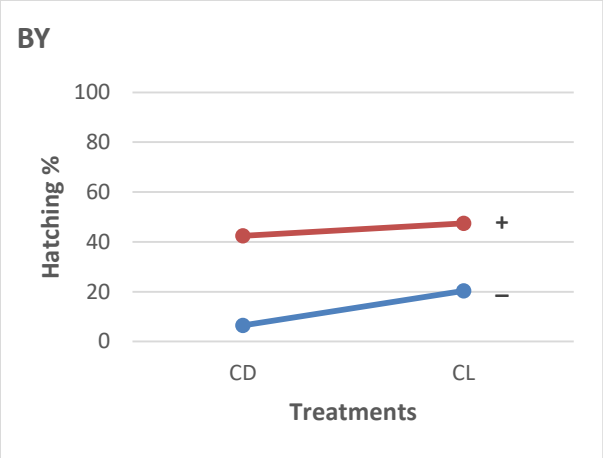








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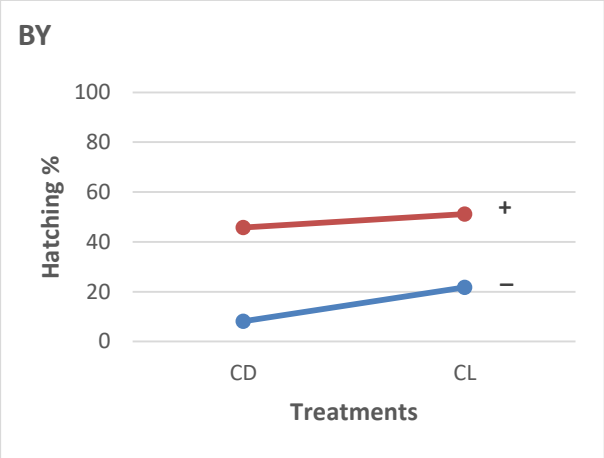
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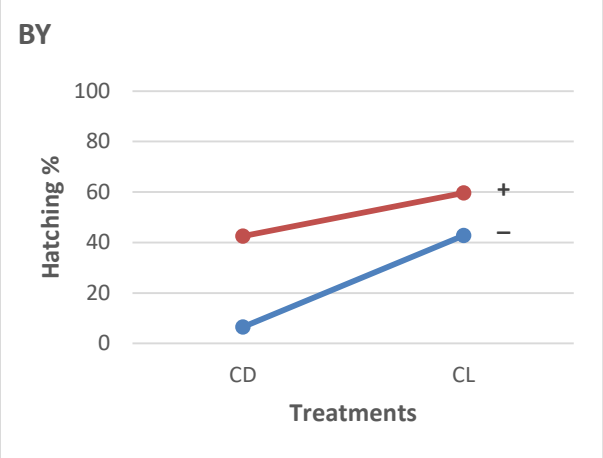
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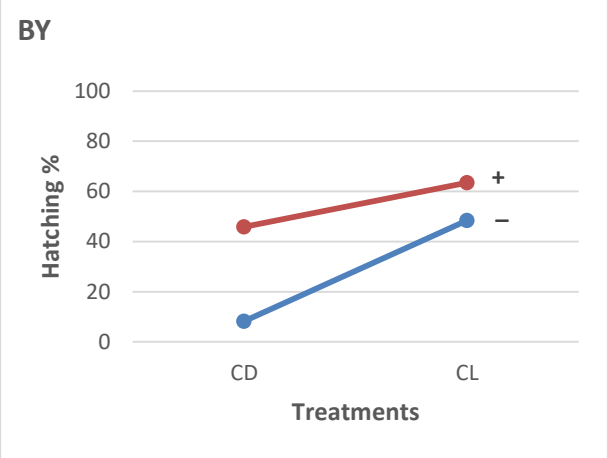
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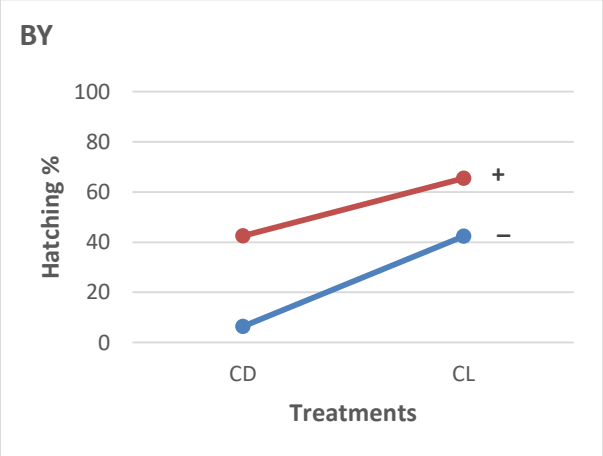
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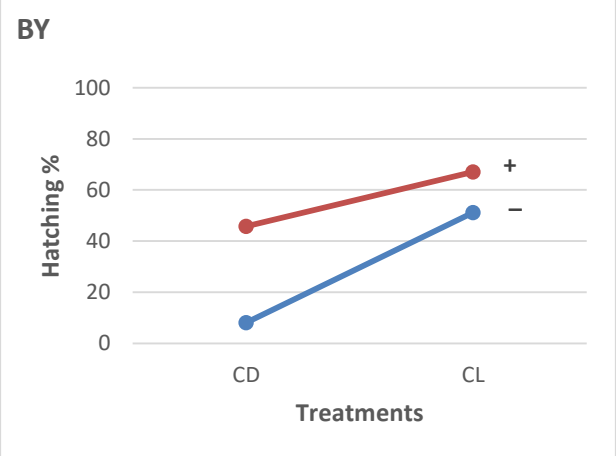
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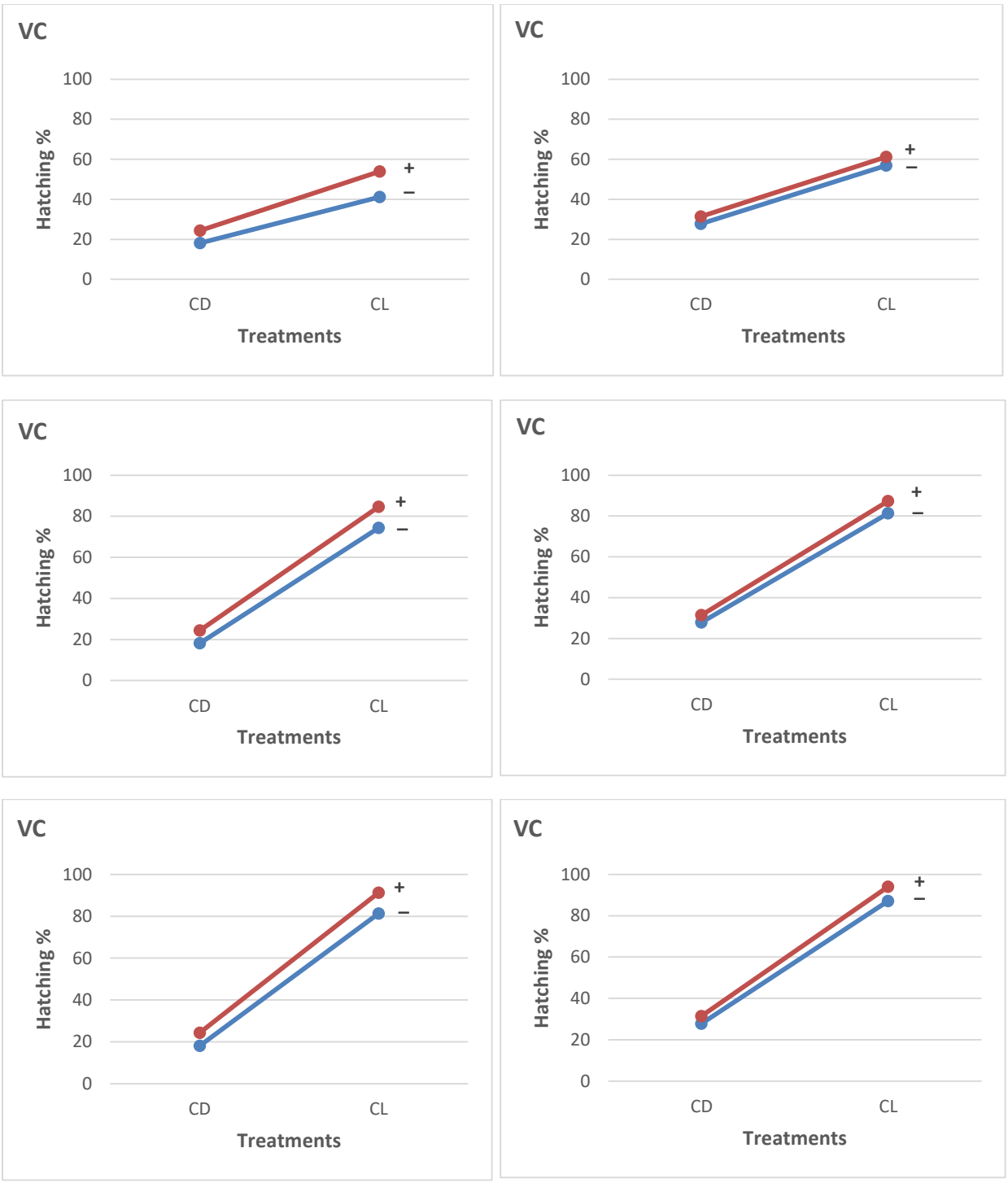
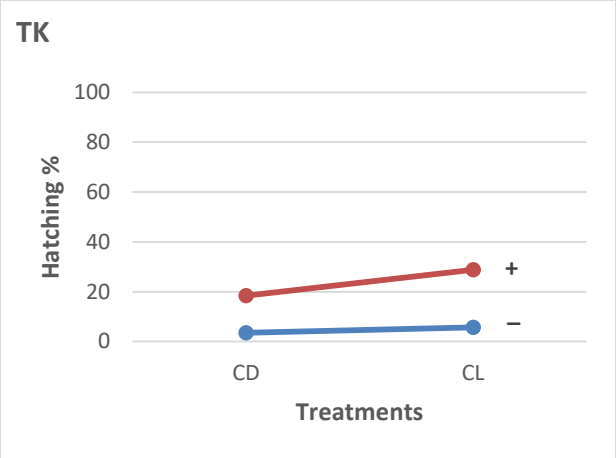


Figure 11.1: Hatching percentage of cysts of **TK**, **BY** and **VC** *Artemia* strains exposed to continuous darkness (CD) and continuous light (CL) in combination with H_2O_2 (+) or without H_2O_2 (-) and incubated for 24 h (left) and 48 h (right). The lines in each figure and for each strain correspond to treatments with red light (top) blue light (middle) and white light (below). Data are mean value (n = 3). TK = Tuz, Kazakhstan. BY = Bolshoye Yarovoye, Russia. VC = Vinh Chau, Vietnam.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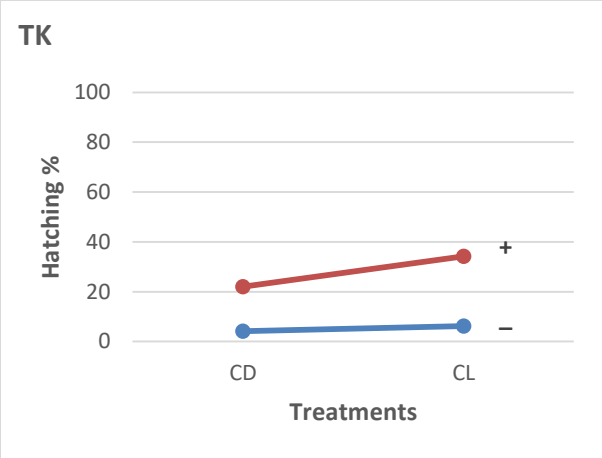
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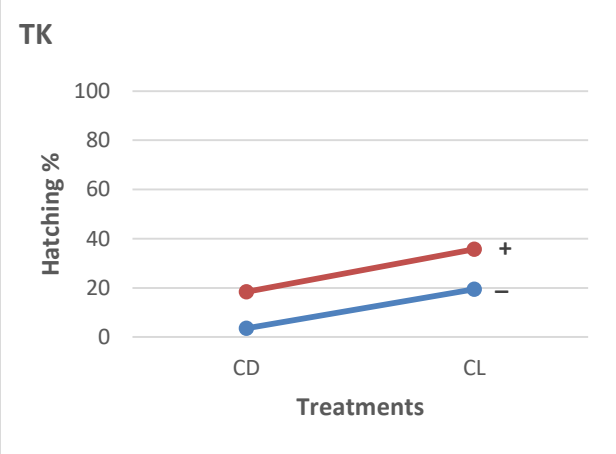
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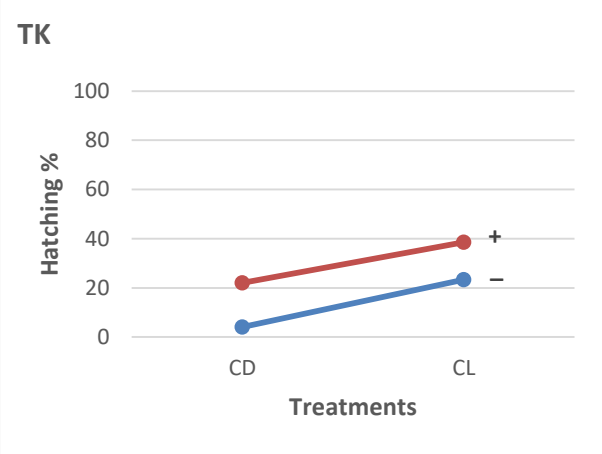
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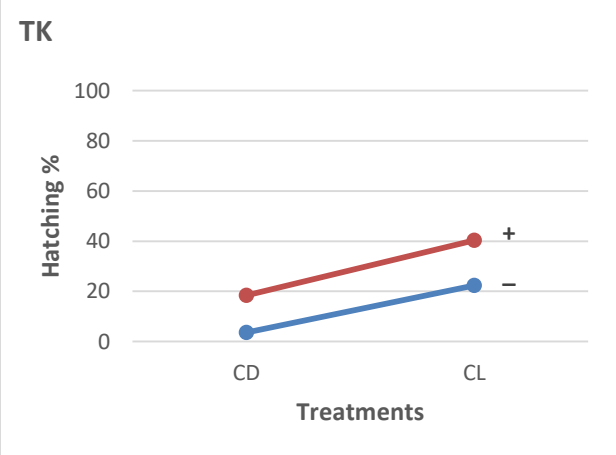
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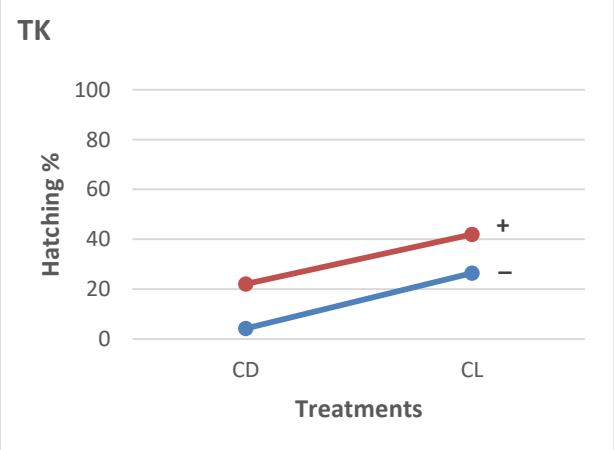
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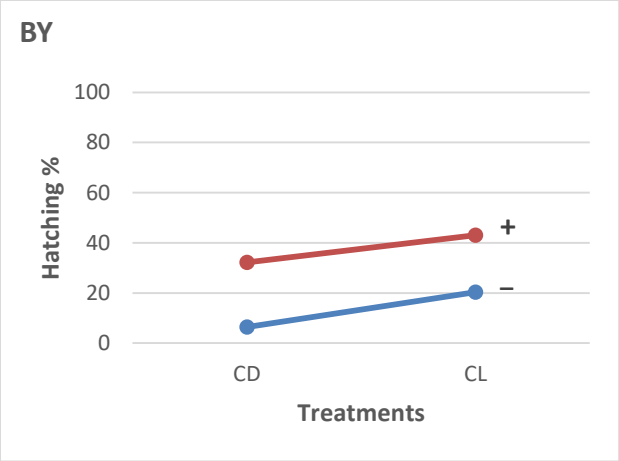
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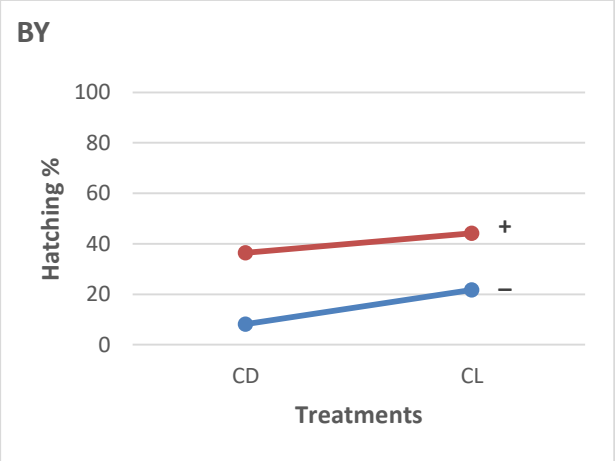
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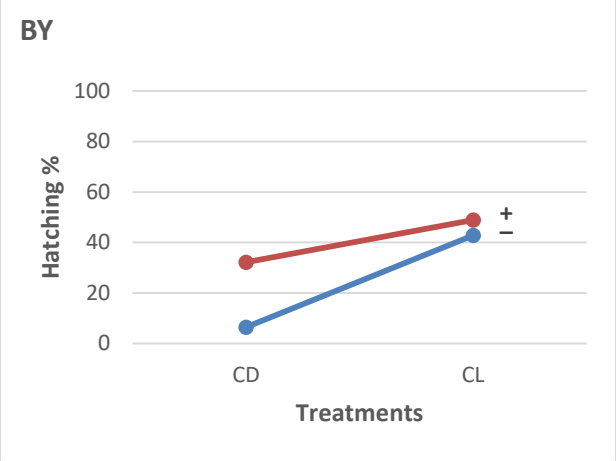
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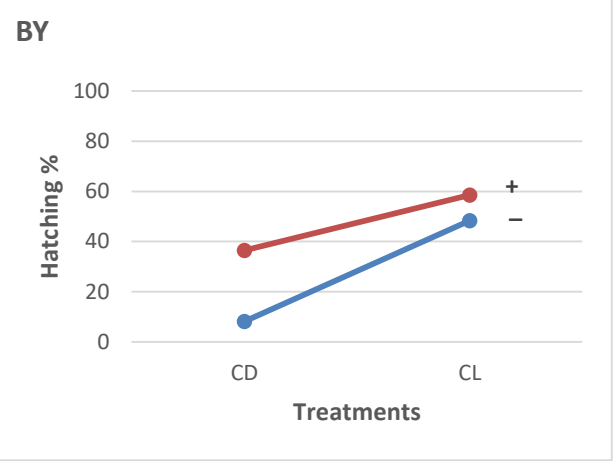
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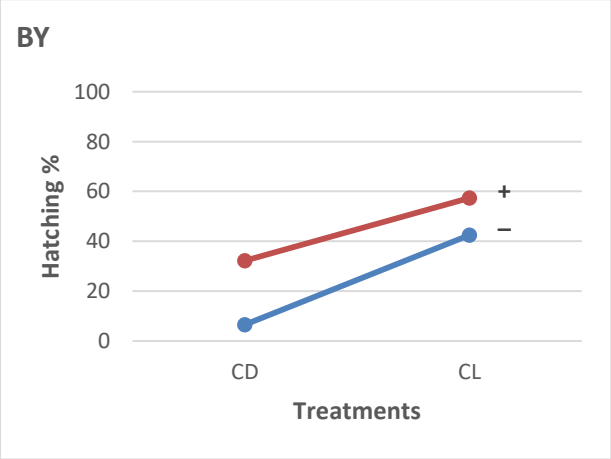
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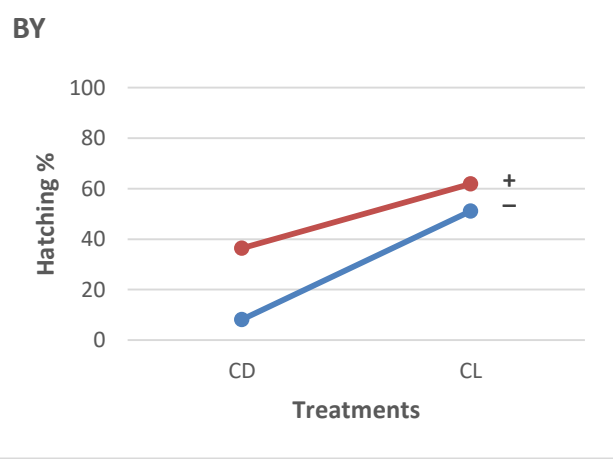
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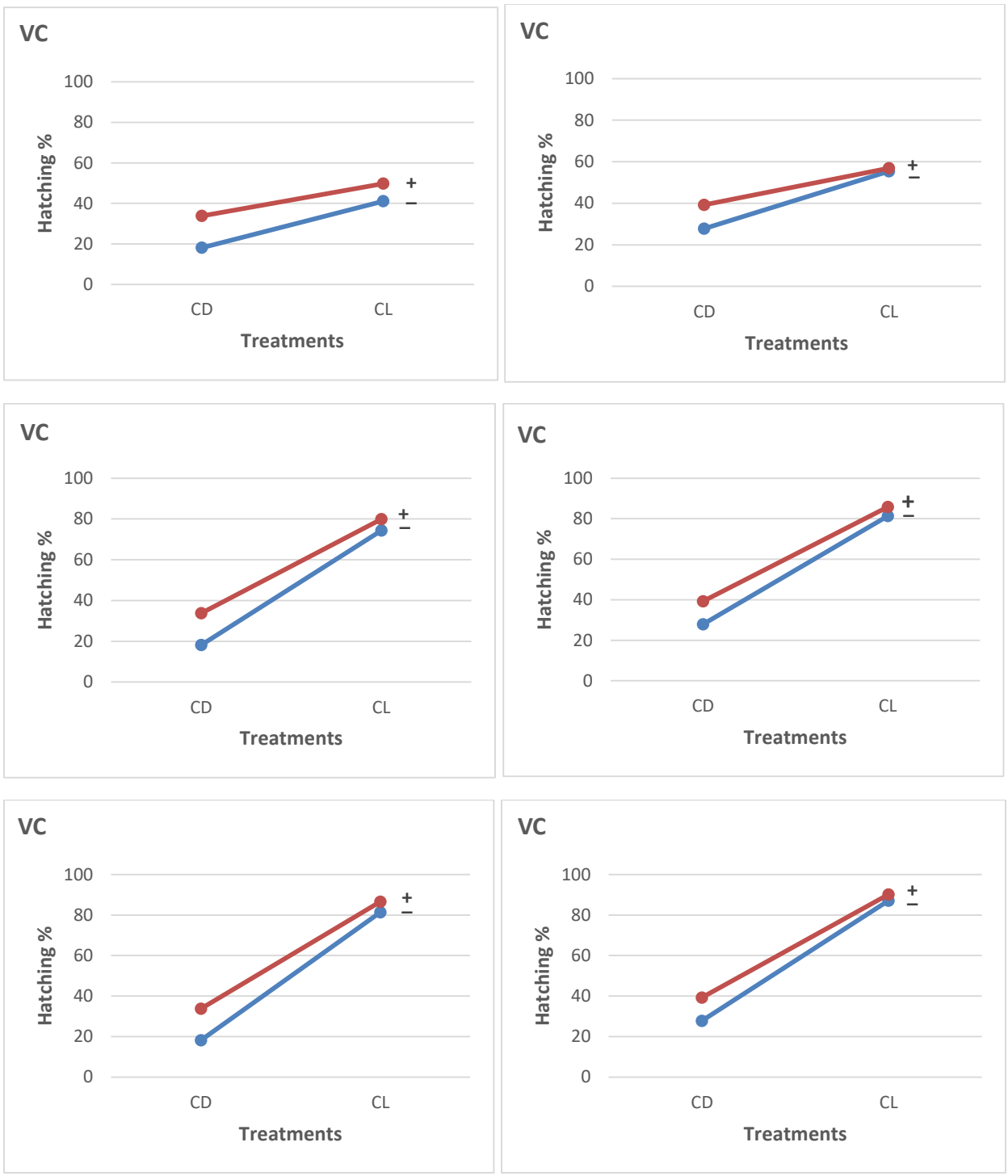

Figure 11.2: Hatching percentage of cysts of **TK**, **BY** and **VC** *Artemia* strains exposed to continuous darkness (CD) and continuous light (CL) in combination with NO (+) or without NO (-) and incubated for 24 h (left) and 48 h (right). The lines in each figure and for each strain correspond to treatments with red light (top) blue light (middle) and white light (below). Data are mean value (n = 3). TK = Tuz, Kazakhstan. BY = Bolshoye Yarovoye, Russia. VC = Vinh Chau, Vietnam.

Appendix G

CURRICULUM VITAE

Personal informations

Name: **Mohamed Omar El-Magsodi**

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Marital status: Married

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P.O.Box: 13538
Tel: + 218-21-4628412
+ 218-21-4628417
Fax: + 218-21-4625100

Educational qualifications

Bachelor Degree in Zoology Science

- 1980/1981 – 1983/1984: Undergraduate study in Department of Zoology Faculty of Science, University of Tripoli,

Master of Science in Aquaculture

- 1996/1997 – 1998/1999: Master study in Laboratory of Aquaculture & *Artemia* Reference Centre, Faculty of Agriculture, Gent University.

Employment Record:

1985 – 2004:	Researcher in Marine Biology Research Center (MBRC) Tajura, Tripoli, Libya
2000 – 2004:	Head of Plankton Department, Marine Biology Research Center (MBRC) Tajura, Tripoli, Libya.
2000 – 2004:	Lecturer collaborator in the Department of Aquaculture, Faculty of Agriculture, University of Tripoli, Libya.
2004 – 2006:	University staff member (as a lecturer) in the Department of Aquaculture, Faculty of Agriculture, University of Tripoli, Libya.

Training Programs Participated

- International training course and workshop on *Artemia*, In: Fisheries Research Center, Suez Canal University, Ismailia, Egypt (duration: 1 month, 1991).

- International *Artemia* and larviculture training course, In: Lab of Aquaculture & *Artemia* Reference Center, Ghent, Belgium (duration: 45 days, 1993).

- International training course in aquaculture, In: National Aquaculture Centre, Malta (duration: 45 days, 1995).

Publication in international peer-reviewed journals

- **El-Magsodi, M.O.,** Bossier, P., Sorgeloos, P. and Van Stappen, G. (2014). Hatching and nutritional quality of *Artemia* cysts progressively deteriorates as a function of increased exposure to hydration/dehydration cycles. Aquaculture International **22:** 1515–1532.

- **El-Magsodi, M.O.,** Baruah, K., Norouzitallab, P. Bossier, P., Sorgeloos, P. and Van Stappen, G. (2016). Hydration/dehydration cycles imposed on *Artemia* cysts influence the tolerance limit of nauplii against abiotic and biotic stressors. Aquaculture International **24(2):** 429–439.

- **El-Magsodi, M.O.,** Bossier, P., Sorgeloos, P. and Van Stappen, G. (2016). Effect of light colour, timing, and duration of light exposure on the hatchability of *Artemia* spp. (Branchiopoda: Anostraca) eggs. Journal of Crustacean Biology **36 (4):** 515–524.

Publications at national level

- **El-Magsodi, M.O.,** (1994). Live feed production: Zooplankton. LIBFISH Technical Briefing notes No. 21. Tripoli/Rome, FAO. pp. 34-35.

- Medina Pizzali, A.F., Vallet, F. and **El-Magsodi, M.O.,** (1994). Proposal for an integrated small-scale solar salt and brine shrimp production unit. LIBFISH Technical Briefing notes No. 17. Tripoli/Rome, FAO. pp.19.

- **El-Magsodi, M.O.,** El-Ghebli, H.M., Hamza, M., Van Stappen, G. and Sorgeloos, P. (2005). Characterization of Libyan *Artemia* from Abu Kammash Sabkha. Libyan Journal of Marine Science **10:** 19-30.

- **El-Magsodi, M.O.,** El-Ghebli, H.M., Enbaya, M.A., Hamza, M., Drebika, U.A. and Sorgeloos, P. (2005). Reproductive and lifespan characteristics of *Artemia* from Libyan Abu Kammash Sabkha. Libyan Journal of Marine Science **10:** 1-8.

- **El-Magsodi, M.O.,** Haddoud, D.A., Dakdak, E.M. and Abdelbari, R. (2006). Distribution of *Artemia* in different Sabkhas along the east coastal part of Libya. Bulletin de l'INSTM N° Spécial 11: Actes des 8èmes Journées des Sciences de la Mer, Hammamet, Tunisie. pp. 129-135.

- Mejri, R., Menif, D., **El-Magsodi, M.O.** and Ben Hassine, O.K. (2007). Nouvelles donnees sur la distribution geographique de *zebrus zebrus* poisson gobiidae (Risso, 1826) au niveau des cotes mediterraneennes meridionales. Rapp. Comm. int. Mer Medit. **38**: 539.

- **El-Magsodi, M.O.** and Haddoud, D.A. (2011). The salt marsh (Sabkha) in the western part of Libya. In: Sabkha Ecosystems: Tasks for Vegetation Science. Öztürk, M. et al. (eds). pp. 79-84.

Publications of scientific reports in different Bulletin of the Marine Biology Research Center (Tripoli, Libya)

- Report on the growth rate of mullet larvae species.

- Report on *Artemia* field work in the middle Libyan coast.
- Report on wadi kaam dam for its utilization in aquaculture.
- Report on the infection of *M. edilus* (mussels) cultured in Farwa lagoon in the western coastal part of Libya.
- Report on aquaculture site selection in the eastern part of Libya.
- Report on the infection of fishes (*common carp* and *silver carp*) cultured in wadi kaam dam.
- Report on the fish dying in Abu Kammash areas in the western coastal part of Libya.
- Report on the biodiversity in the Libyan coast.
- Report on marine protected areas along the Libyan coast.
- Report on ecological study of Ain Gazala lagoon and its fitness for aquaculture.
- Report on integrated small-scale solar salt and brine shrimp production unit in Abu Kammash areas in the western coastal part of Libya.
- Report on planning for aquaculture development in Libya.
- Report on the Sabkha ecology in the western part of Libya.
- Report on the geographical distribution of *Artemia* in Libya.

Thesis supervision

- Supervisor of more than 10 thesis of students pursuing studies towards the degrees of B.Sc. in Department of Aquaculture, Faculty of Agriculture, University of Tripoli, Libya

Participation in scientific mission, conferences, symposia, workshops and seminars

- Participation in more than 20 different scientific missions include: conferences, symposia, workshops and seminars inside and outside Libya on marine biology and aquaculture field.

Appendix H

ACKNOWLEDGEMENTS

It is my duty to express my sincere thanks to all who were behind me in successful completion of this study.

First of all, I would like to thank the Libyan government (Ministry of Higher Education) for providing me a scholarship to pursue my PhD study at Ghent University, Belgium.

Secondly, I would like to extent my special gratitude to my first promoter Prof. Dr. Gilbert Van Stappen, for his excellent guidance throughout my doctoral studies in Ghent University. During the days in the *Artemia* Reference Center (ARC), I have learnt enormously and improved significantly in my scientific abilities. His constant motivation has helped me build-up the passion for science and I truly thank him for his kind suggestions on the experimental design, useful comments and the many scientific discussions. Also, my sincere appreciations for his constant support, understanding and patience especially during the very stressful moments of my research work. I sincerely thank his tireless in correcting my papers and thesis. Truly, without his assistance, this PhD could never come to the end. It was a great honor to work with him. *Thank you very much sir, being such a wonderful teacher.*

I am gratefully beholden to Prof. dr. ir. Peter Bossier, my second promoter, for his enthusiastic guidance, encouragement and support during my PhD study, especially his patience and tireless during the very stressful moments of my research work and also his useful comments and the many scientific discussions. It is true that without his precious and constructive comments given on my research articles none of my work would success. I thank you very much for your kind contributions and finding times to review my manuscripts in despite of your busy schedule.

I also express my deep gratitude to Em. Prof. Dr. Patrick Sorgeloos for giving me the opportunity to study my PhD at the Laboratory of Aquaculture & *Artemia* Reference Center, Ghent University. I never forget the strength and kindness given to me as well as to my family by you and by your lovely wife, Magda, who passed away suddenly. I will never forget her helps and sympathy for foreign students to overcome the difficult situations in both academic as well domestic lives. I hereby send my deepest sympathy to her family. God bless her.

Big thanks to all the ARC staffs, Dr. Tom Defoirdt, Dr. Peter De Schryver, Jean Dhont, Dr. Nancy Nevejan, Mieke Eggermont, Aäron Plovie, Mathieu Wille, Tom Baelemans, Anita De Haese, Jorg Desmyter, Christ Mahieu, Alex Pieters, Geert Vandewiele, Caroline Van Geeteruyen, Brigitte Van Moffaert, Marc Verschraeghen, and others, for their constant support in solving all the administrative issues and also for their assistance in carrying out the experiments. I never forget generous supports of Christ Mahieu and Tom Baelemans for providing me chemicals and materials I needed for my experiments. I am greatly indebted to Geert Vandewiele for fatty acid and vitamin C analysis of the *Artemia* samples.

I am especially thankful to all members of the examination and reading committee for sharing their experience, knowledge, critical reviews and extremely valuable suggestions to improve the scientific quality of this thesis.

Special thanks also to my best friend Dr. Kartik Baruah and Dr. Parisa Norouzitallab for their challenges, discussions and support during my study. I will never forget their helps and sympathy.

Many thanks to all my friends and my PhD colleagues: Sunny, Asanka, Ana Maria, Cam, Phouc, Ebrahim, Ramin, Michael, Bing, Eamy, Pande, Toi, Lenny, Spyros, Sofie, Stephanie, Ruy Santos,

Li Shi Hao, Han Biao, Nguyen Van Hung, Nguyen Thi Xuan Hong, Nguyen Viet Dung, Thi Minh Tho Dam for challenges, discussions and help during my study.

My warmest thanks to all my friends and my colleagues in the department of Aquaculture, faculty of Agriculture, University of Tripoli and also the Marine Biology Research Center, Tajura, Tripoli, who always were concerned about my PhD completion.

I would like to dedicate this thesis to the spirit of my mother and father who passed away suddenly. I will never forget their prayers, support and helps to overcome the difficult situations in both academic as well domestic lives. God blesses them.

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